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June 28, 2004

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

A. DRAV

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Elimelech	ROCHLIN	D.N. Shimshon, ISRAEL

☑ Additional inventors are being named on the 2nd separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Sphingolipids' Polyamines Conjugates Mediated Delivery Systems

CORRESPONDENCE ADDRESS

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PATENT TRADEMARK OFFICE

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ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification	Number of Pages: 42	☐ CD(s), Number	
☑ Drawing(s)	Number of Sheets: 10	☑ Other (specify)	transmittal letter, postcard
☐ Application Data Sheet. See 37 CFR 1.76			

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

		FILING FEE AMOUNT ($)
☑ Applicant claims small entity status. See 37 CFR 1.27.		
☑ A check or money order is enclosed to cover the filing fees		
☑ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:	14-0112	$80.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ Date 6/18/2003

TYPED or PRINTED NAME Todd L. Juneau / Joshua B. Goldberg REGISTRATION NO. (if appropriate) 40,669 / 44,126

TELEPHONE 202-775-8383 Docket Number: 25513

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	25513

INVENTOR(S)/APPLICANT(S)		
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Number 2 of 2

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MAIL STOP, PROVISIONAL PATENT APPLICATION
Attorney Docket No. 25513

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Elimelech ROCHLIN et al.

Serial No. NOT YET ASSIGNED

Filed: June 18, 2003

For: **SPHINGOLIPIS' POLYAMINES CONJUGATES MEDIATED DELIVERY SYSTEMS**

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
PPP
Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing Provisional Application;
(3) 52 page Provisional Application consisting of:
28 pages Textual Specification,
4 pages of claims,
1 page of the abstract,
9 pages of tables,
10 sheets drawings;
(4) Check No. 18798 $ 80.00 for filing fee as a small entity;
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-011.

Respectfully submitted,
NATH & ASSOCIATES PLLC

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SPHINGOLIPIDS' POLYAMINES CONJUGATES MEDIATED DELIVERY SYSTEMS

FIELD OF THE INVENTION

The present invention concerns vehicles for effective delivery of biologically active agents, particularly polynucleotides, proteins, peptides, and drug molecules, by facilitating transmembrane transport or by encouraging adhesion to biological surfaces and/or affecting intracellular traffic and processing of the biologically active agent as well as affecting the cells interacting by the delivery system to better respond biologicallly.

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BACKGROUND OF THE INVENTION

Many natural biological molecules and their analogues, including proteins and polynucleotides, foreign substances and drugs, which are capable of influencing cell function at the sub-cellular or molecular level are preferably incorporated within the cell in order to produce their effect. For these agents the cell membrane presents a selective barrier which is impermeable to them. The complex composition of the cell membrane comprises phospholipids, glycolipids, and cholesterol, as well as intrinsic and extrinsic proteins, and its functions are influenced by cytoplasmic components which include Ca^{++} and other metal ions, anions, ATP, microfilaments, microtubules, enzymes, and Ca^{++}-binding proteins.

Interactions among structural and cytoplasmic cell components and their response to external signals make up transport processes responsible for the membrane selectivity exhibited within and among cell types.

Successful intracellular delivery of agents not naturally taken up by cells has also been investigated. The membrane barrier can be overcome by associating agents in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. These formulations may fuse with the cell membranes on contact, and in these processes, the associated substances are delivered intracellularly (adsorptive endocytosis).

Lipid complexes can facilitate intracellular transfers also by overcoming charge repulsions between the cell surface, which in most cases is negatively charged, and the molecule to be inserted. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form various layers or aggregates such as micelles or hollow lipid vesicles (liposomes), in aqueous systems. The liposomes can be used to entrap the substance to be delivered within the liposomes; in other applications, the drug molecule of interest can be incorporated into the lipid vesicle as an intrinsic membrane component, rather than entrapped into the hollow aqueous interior, or electrostatically attached to aggregate surface.

An advance in the area of intracellular delivery was the discovery that a positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, could interact spontaneously with DNA to form lipid-DNA complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in both uptake and expression of the DNA (Felgner, P.L. et al., Proc. Natl. Acad. Sci. USA 84:7413-7417 (1987) and U.S. Patent No. 4,897,355 to Eppstein, D.E. et al.). Others have successfully used a DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) in

combination with a phospholipid to form DNA-complexing vesicles. The Lipofectin™ reagent (Bethesda Research Laboratories, Gaithersburg, Md.), an effective agent for the delivery of highly anionic polynucleotides into living tissue culture cells, comprises positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When excess of positively charged liposomes over DNA negative charges are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces or introduced into the cells either by endocytosis or fuse with the plasma membrane, both processes deliver functional polynucleotide into, for example, tissue culture cells.

Multivalent cations by themselves (including polyamines, inorganic salts and complexes and dehydrating solvents) have also been shown to facilitate delivery of macromolecules into cells. In particular, multivalent cations provoke the collapse of oligo and polyanions (nucleic acids molecules, amino acid molecules and the like) to compact structural forms, and facilitate the packaging of these polyanions into viruses, their incorporation into liposomes, transfer into cells etc. (Thomas T.J. et al. Biochemistry 38:3821-3830 (1999)). The smallest natural polycations able to compact DNA are the polyamines spermidine and spermine. By attaching a hydrophobic anchor to these molecules via a linker, a new class of transfection vectors, the polycationic lipopolymers, has been developed.

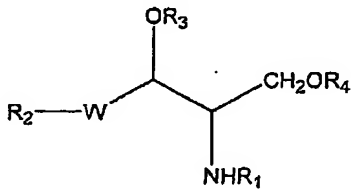
Cationic lipids and cationic polymers interact electrostatically with the anionic groups of DNA (or of any other polyanionic macromolecule) forming DNA-lipid complexes (lipoplexes) or DNA-polycation complexes (polyplexes). The formation of the complex is associated with the release of counterions of the lipids or polymer. The cationic lipids can be divided into four classes: (i) quaternary ammonium salt lipids (e.g. DOTMA (Lipofectin™) and DOTAP) and phosphonium/arspnium congeners; (ii) lipopolyamines; (iii) cationic lipids bearing

both quaternary ammonium and polyamine moieties and (iv) amidinium, guanidinium and heterocyclic salt lipids.

SUMMARY OF THE INVENTION

The present invention is based on the fact that novel compounds may serve as efficient vehicles for the delivery of polynucleotides, oligonucleotides, proteins, peptides and drugs into cells by facilitating their transmembrane transport.

Thus the present invention is directed to a compound of formula (I):



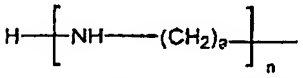
wherein

R_1 is selected from hydrogen, branched or normal alkyl, aryl, lower alkyl amine wherein said amine may be substituted, or $C(O)R_5$

W is $-CH=CH-$, $-CH_2-CH(OH)-$ or $-CH_2-CH_2-$,

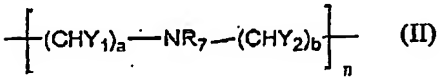
R_2 and R_5 are independently selected from a saturated or unsaturated normal or branched C_{10}-C_{24} alkyl, alkenyl or polyenyl groups, preferably from C_{12}-C_{18}; and most preferably C_{12}-C_{16};

R_3 and R_4 are independently selected from hydrogen, a group –C(O)-NR_6X-Z, Z being a same or different for R_3 and R_4; or R_3 and R_4 form together with the oxygen atoms to which they are bound a heterocyclic group comprising –C(O)-NH–Z–NH-C(O)–; R_6 is hydrogen or



wherein a represents an integer from 1 to 4; n represents an integer from 1 to 6 preferably from 1 to 3;

Z being a branched or linear polyalkylamine chain wherein said polyalkylamine chain may comprise quaternary ammonium groups; with the proviso that R_3 and R_4 are not simultaneously hydrogen. The polyalkylamine chain is of formula (II):

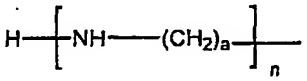


(II)

wherein a and b are independently selected from 1, 2, 3 or 4;

n represents an integer from 1 to 6, preferably from 2 to 4 ;

R_7 is selected from hydrogen or



where a and n have the meaning as above; and Y_1 and Y_2 are independently selected from hydrogen and alkyl.

Lower alkyl is defined as a C_1-C_5-alkyl group which may be linear or branched and which may substituted.

The present invention is further directed to a process for preparing a compound of formula (I).

The invention is further directed to a method for introducing biologically active compounds into eukaryotic cells comprising forming liposomes from the compound of formula (I), contacting said liposomes with a biologically active compound to form a complex and administering said complex.

The invention is yet further directed to a complex comprising liposomes formed from the compound of formula (I) and a biologically active compound. The biolgically active compound is selected from polynucleotides, oligonucleotides, proteins, peptides and drugs. Administration of polynucleotides and oligonucleotides may be used in gene therapy, gene vaccination and transfection. Proteins, peptides and drugs may be used in new methods of vaccination and drug delivery.

The invention is yet further directed to pharmaceutical compositions comprising the complex comprising liposomes formed from the compound of formula (I) and a biologically active compound together with suitable excipients. The pharmaceutical composition may be in the form suitable for intravenous, subcutaneous, topical, intranasal, oral, ocular, intramuscular administration.

BRIEF DESCRIPTION OF THE FIGURES

In order to understand the invention and to see how it may be carried out in practice, some embodiments will now be described, by way of non-limiting examples only, with reference to the accompanying figures, in which:

Fig. 1 shows several possible chemical structures, "linear", branched" or "cyclic" compounds according to the present invention.

Fig. 2 Shows the Mass Spectrum of a specific compound (3-amino-propyl) – [4-(3-amino-propylamino)-butyl]-carbamic acid –hexadecanoylamino-3-hydroxy-octadec-4-enyl ester (chemical structure shown). The Expected M.W. is 766. Elementary analysis: C – 6.5%; H-11.65%, N-8.01%. TLC system: Chloroform:Methanol: Acetic acid 1:25:1.5. TNBS-reactive primary amines: 2 per molecule. Solubility: ethanol (base).

Fig. 3 shows growth of MCF-7 cells following application of (A) LUV (large unilamellar vesicles)-derived or (B) HV(heterogeneous vesicles)-derived lipid-like cationic (LLC)- assemblies. Throughout, [S-ODN] = 0.1 μM and [L+]/[S-ODN] = 2. Each error bar represents the s.e.m. where n (no of wells) ≥ 9. The data were derived from experiments performed on 3 separately cultivated cell batches.

Fig. 4 shows Western blots for quantification of bcl-2 in MCF-7 cells. (A) Bcl-2 western blot in MCF-7 cells following application of various treatments. (B) Corresponding densitometric analysis of bcl-2 western blot in MCF-7 cells.

Fig. 5 shows MCF-7 cell growth as a function of Genofect™ lipoplex (HV-derived) concentration. Throughout, [L+]/[ODNP-] = 2. Each error bar represents the s.e.m. where n (no of wells) ≥ 9. The data were derived from experiments performed on 3 separately cultivated cell batches.

Fig. 6 shows MCF-7 cell growth as a function of lipid-like (LLC) lipoplex (HV-derived) charge ratio. Throughout, [S-ODN] = 0.1 μM. Each error bar represents the s.e.m. where n (no of wells) ≥ 9. The data was derived from experiments performed on 3 separately cultivated cell batches.

Fig. 7 shows toxicity studies on NIH 3T3 cells revealing that the lipid-like cationic (LLC) vehicles (B5C – certain batch in the form of a chloride salt) are non toxic to cells.

Fig. 8 shows gene transfection of luciferase plasmid into NIH 3T3 cells. Transfection was carried by lipid-like cationic (LLC) vehicles together with DOPE.

Fig. 9 shows a comparison of transfection of C-26 cells with luciferase: a comparison between lipid-like cationic (LLC) vehicles and FuGENE6®.

Fig. 10 shows a pictorial comparison of the efficiency of transfection of pGFP into cells using the lipid-like cationic (LLC) vehicles of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns novel vehicles for delivering of polynucleotides, oligonucleotides, proteins, peptides and drugs into cells. The vehicles are lipid-like cationic (LLC) compounds, which may be synthesized in the following manner. N-substituted long-chain bases in particular, N-substituted sphingoids or sphingoid bases are coupled together with different polyamines or their derivatives, to form a polyamine-sphingoid entity, which is used as is or further alkylated. Protonation at a suitable pH or alkylation of the formed polyamine-sphingoid entity attributes to the lipid-like compounds the desired positive charge for interaction with both the active biological entities to be

delivered into the target cells and with the targeted cells. The formed lipid-like compounds, after their synthesis may be directly complexed efficiently with biological entities in the form of anions or polyanions to form complexes (lipoplexes). Alternatively, prior to their complexation they are converted into vesicles. Non limiting examples of such biological entities are polynucleotides, oligonucleotides, proteins, peptides and drugs. Thus the formed complex is suitable for targeting such active biological entities into cells. Non-limiting examples of the sphingoids or sphingoid bases are sphingosine, dihydrosphingosine, phytosphingosine, dehydrophytosphinosine or derivatives thereof. Non-limiting examples of such derivatives are acyl derivatives, such as ceramide (N-acylsphingosine), dihydroceramides, phytoceramides and dihydrophytoceramides, respectively. The suitably N-substituted sphingoids or sphingoid bases, in addition to the free amine which may be derivatized, posses free hydroxyl groups which are activated and subsequently reacted with the polyamines to form the polyamine-sphingoid entity. Non-limiting examples of activation agents are N,N'-disuccinimidylcarbonate, di- or tri-phosgene or imidazole derivatives. The reaction of these activation agents with the sphingoids or the sphingoid bases yields a succinimidyloxycarbonyl, chloroformate or imidazole carbamate, respectively, at one or both hydroxyls. The reaction of the activated sphingoids with polyamines may yield branched, normal (unbranched) or cyclic compounds. **Fig. 1** displays chemical structures of several possible compounds prior to their quaternization. **Fig. 2** further displays the Mass Spectrum of one specific compound, ceramide coupled with spermine. Formation of branched, normal or cyclic polyamine sphingoid may be directed by monitoring the excess of polyamine used in the reaction and suitable protection of polyamine prior to use. The formed conjugates of the sphingoids with the polyamines could be further reacted with methylation agents in order to form quaternary amines. The resulting compounds are positively charged to a different degree depending on the ratio between the quaternary, primary and/or secondary amines within the formed

conjugates. The resulting non-methylated or methylated lipid-like compounds may then be processed by any known method to form liposomes. Such processing may include (non-limiting examples) incorporation of different non-cationic lipids like DOPE, Cholesterol or others at different mole ratios to the lipid-like compound. The formed liposomes may be shaped as heterogeneous vesicles (HV) having a diameter of about 500 – 5000 nm. The formed HV, may be downsized and converted to large unilamellar vesicles (LUV) having a diameter of about 100 nm by further processing. The structure and dimensions of the vesicles, e.g. their shape and size may have important implications on their efficiency as vehicles for delivery of the active biological entities to the target, i.e. these determine their transfection properties. Thus the structure of the formed vesicles, HV or LUV, is one important factor. Another important factor for efficient delivery is the ratio between the amount of the amine positive charge of the lipid-like (L^+) and the negatively charged oligo or polyanion complexed therein (or on the lipid-like surface) (A^-). The ratio determines the overall charge of the charged complex, where for effective delivery the ratio may be $0.1<L^+/A^-<1000$ depending on the entrapped moiety. In case of a particular oligonucleotide of the present invention, the ratio was found to be $1< [L^+/ODN^-]< 2$.

Such transfection facilitates for example mucosal vaccination, introduction of genes into cells for their expression, gene and oligo- and poly - nucleotide therapy. In addition, the lipid-like cationic liposomes are able to efficiently induce on mice peritoneal macrophage surface the expression of high levels of MHC II and costimulatory molecules such as B7 and CD40. These are essential for productive antigen presentation. Neutral and anionic liposomes do not possess such abilities. This can explain the unique and superior adjuvant activity of the formulations of the present invention. Thus the formed complexes may be part of a pharmaceutical composition comprising the complex bearing the active biological entity to be delivered together with suitable excipients. Such a pharmaceutical

composition may be prepared such that it may be administered intravenously, subcutaneously, topically, intranasaly, orally, ocularly or intramuscularly.

One important example of efficient use of the lipid-like cationic (LLC) vehicles is in oligonucleotide transfer into cells, and in particular into cancerous cells. One approach in cancer treatment is to target specified poly- or oligonucleotides in the form of antisense in order to interfere with cancer cell function. Many types of tumors overexpress bcl-2, a key apoptosis regulating protein that has been implicated in mechanisms of chemoresistance by blocking programmed cell death. An 18-mer antisense phosphorothioate oligonucleotide (S-ODN) targeted to the first 6 codons of the open reading frame of bcl-2 was designed (Genta Inc.). In order to achieve efficient treatment, the antisense oligonucleaotide should be transferred into cancerous cells. Such targeting requires an appropriate vehicle. Commercial lipids and lipid-like compounds synthesized according to the present invention were used in order to find the optimal complex, which delivers the S-ODN in a most efficient manner. DOTAP, DOPE, DOPC, cholesterol and their mixtures were used to form lipoplexes and liposomes. These commercial liposomes and lipoplexes were compared with the lipid-like compounds according to the present invention. **Fig. 3** shows the results of experiments performed with lipid-like cationic lipoplexes and liposomes based on commercial lipids. As can be seen from **Fig. 3A**, when MCF-7 breast cancer cells were incubated with LUV-derived lipid assemblies, cell growth was either unaffected or very marginally reduced. **Fig. 3B** presents the influence of HV-derived complexes and vesicles. In this case, treatment with all LLC/DOPC assemblies, or all LLC/DOPE assemblies or LLC liposomes induced a negligible effect on cell growth. However, while incubation of MCF-7 cells with LLC-reverse sequence (of the antisense 18-mer) lipoplexes produced a marginal (14%) reduction in cell growth, incubation with LLC-S-ODN complexes decreased cell growth to a large extent (50%). Moreover, this difference was highly significant (Student's t-test: $P<0.001$). Confirmation of the direct involvement of bcl-2 in cell growth inhibition was accomplished by the use of a

Western blot technique to quantify bcl-2 protein expression. Fig. 4A and Fig. 4B, respectively, show the Western blot results and the corresponding bcl-2/actin ratio values derived from densitometric analysis. The densitometry clearly reveals that while incubation with S-ODN- LLC lipoplexes reduced cellular bcl-2 levels by some 90%, incubation with the other control treatments *i.e* LLC liposomes, reverse sequence- LLC lipoplexes or free S-ODNs, exerted a negligible or marginal influence on cellular bcl-2 levels. These results indicate that the delivery of S-ODN as MLV-derived LLC lipoplexes, facilitates enhanced antisense downregulation of bcl-2. In fact, by using this lipid vector with 0.1 μM G3139, cell growth was reduced by approximately the same extent as using free G3139 at a concentration of 5 μM (data not shown). Thus, antisense efficacy was improved 50-fold by using the LLC lipoplex of the present invention.

The efficiency of the LLC/S-ODN depends on the effective concentration of LLC/S-ODN and on ratio between the S-ODN and LLC entity. Since the LLC is positive, while the S-ODN (like any polynucleotide) is negatively charged, the latter concerns the charge balance of the formed complex.

In order to determine the concentration dependence, lipoplexes were prepared at a fixed L^+ to $ODNP^-$ mole ratio (charge ratio) of 2. The data is presented in Fig. 5. It can be seen that at an S-ODN concentration of 0.05 μM, all the lipid assemblies exerted a negligible or marginal effect on cell growth. However at a concentration of 0.2 μM, exposure to both S-ODN lipoplexes and reverse sequence lipoplexes suppressed cell growth. This indicates that enhanced antisense activity by these complexes develops only at a very narrow ODN concentration "window" around 0.1 μM. If the concentration of complexes is slightly reduced below this value, the complexes exert no effect while if complex concentration is slightly elevated above this value they exert a non-specific cellular toxicity. In order to ascertain the optimal ratio between the S-ODN and its vehicle, concentration was maintained at 0.1 μM and MCF-7 cell growth was assessed as a function of the L^+ to S-ODN (which is actually $S\text{-}ODNP^-$ since the phosphate

groups of the oligonucleotide determine its negative charge) mole ratio of HV-derived lipid-LLC assemblies. It can be seen from Fig. 6 that only complexes formed at L^+ / $ODNP^-$ mole ratio of 1 or 2 facilitated enhanced antisense efficiency. More positively charged or negatively charged lipoplexes were ineffective in downregulating bcl-2.

Gene transfection into cells is another utilization of the lipid-like vehicles of the present invention. Fig. 7 clearly demonstrates that the LLC vehicles of the present invention (in particular, the chloride salt of positively charged amine groups) are not toxic when tranfected into NIH 3T3 cells. Turning to Fig. 8, effective transfection of luciferase into NIH 3T3 cells is demonstrated. The vehicle used was a mixture of the LLC vehicles together with the commercial lipid DOPE. A comparison of transfection into C-26 cells using LLC vehicles and FuGENE6® is shown in Fig. 9. Fig. 10 further shows a pictorial demonstration of the efficiency of transfection of pGFP into NIH 3T3 cells using a mixture of LLC vehicles/DOPE in a 2:1 ratio.

Another use of the lipid-like cationic vehicles is in vaccination, in particular mucosal vaccination. Although most pathogens use the mucosal routes (respiratory, gastrointestinal, urogenital) for invasion, the majority of the currently available vaccines are administered parenterally, usually i.m. Parenteral immunization has been found to be very effective in evoking the systemic immune response, however, local immunity at the portals of pathogen entry is often suboptimal with injectable vaccines. In contrast, potent mucosal vaccines can trigger both local and systemic immunity, and mucosal immunity may be less affected by aging compared with systemic immunity. Moreover, since needle-free mucosal vaccines can be self-administered, compliance would be greater, and large populations could be immunized within a short period of time.

A major problem with mucosal (e.g., intranasal, oral) vaccines consisting of killed microorganisms or their purified products is poor immunogenicity. Unless fortified with strong adjuvants, such vaccines may even induce tolerance.

Unfortunately, some of the most powerful adjuvants in animals, such as cholera toxin (CT) or the *E. Coli* enterotoxin are too toxic and are not approved for human use.

The immunostimulating activity of antigen-loaded liposomes is attributed to: (a) protection of the entrapped antigen against degradation or neutralization, (b) slow release of the antigen over an extended period of time (depot effect), and (c) efficient uptake by antigen-presenting cells (APC) such as dendritic cells and macrophages. Two injectable liposomal vaccine formulations, against hepatitis A and influenza, are already in use in humans.

The lipid-like cationic vehicles of the present invention, when loaded with the appropriate antigen(s) and optionally with an adjuvant and administered intranasally (i.n.), are highly effective in inducing strong humoral and cellular, local and systemic immune responses, and protective immunity, against the influenza virus. Intranasal administration of the loaded lipid-like cationic liposomes elicited robust local (in the nose and lungs) and systemic (in the serum) hemagglutination inhibition (HI), IgG1, IgG2a and IgA responses, cytotoxic activity, IFNγ production, and protective immunity. Cationic formulations, composed of both commercially known and the lipid-like cationic vehicles of the present invention, when given i.n., were up to 10,000 times more efficacious than the non-liposomal vaccine or liposomal vaccine formulations comprising neutral and anionic lipids.

In order to evaluate the optimal composition most efficient for vaccination, various liposomal vaccine formulations were compared for antigen encapsulation efficiency and immunogenicity, using varying lipid/antigen w/w ratios (with and without addition of cholesterol) and instillation volumes, and with and without an adjuvant. In addition, the induction of humoral and cellular responses by various routes (intranasal, intramuscular, oral) and the effect of each route on the immune response and protective immunity to the live virus challenge were also tested. The results are given in Tables 1-8.

HN Antigen encapsulation

Efficiency of HN (a commercial preparation of hemagglutinin and neuraminidase derived from influenza A virus) encapsulation in the various liposomal formulations at different lipid/protein w/w ratios (3/1-300/1), and with and without cholesterol (Chol) was tested.

The percentage of encapsulation in all the neutral, anionic and cationic liposomes (Example 5) was 75-90% using a lipid/protein w/w ratio of 50/1 to 300/1, with and without Chol. Table 1 shows the results of an experiment in which the liposomes consisted of the cationic lipid DOTAP. At lipid/protein w/w ratios of 30/1 to 300/1, ~90% antigen encapsulation was achieved, decreasing to 79% and 35% at 10/1 and 3/1 w/w ratios, respectively. The addition of Chol to the formulation did not affect encapsulation at DOTAP/Chol mole ratios of 1/1 and 2/1, with slightly lower encapsulation (80%) at a ratio of 4/1.

HN association with the liposomes upon simple mixing of the soluble antigen with preformed liposomes was also determined. In such cases, 40-60% of the antigen was associated with the liposomes, regardless of the formulation.

These finding, collectively, indicate very high encapsulation efficiency using a simple and fast (5 min.) procedure in all formulations. Furthermore, even preformed liposomes in aqueous suspension are capable of effectively associating with the influenza virus surface antigens.

Immunogenicity of HN-loaded neutral, anionic and cationic liposomes (Lip) administered intranasally

In the first experiment, a comparison was made between neutral (DMPC), anionic (DMPC/DMPG, 9/1 mole ratio) and cationic (6 formulations) liposomes (Lip) encapsulating the HN antigens to induce local and systemic responses following two i.n. administrations. For all formulations, the lipid/HN w/w ratio was 300/1, and the cationic lipid/Chol or cationic lipid/DOPE mole ratio was 1/1.

Free antigen (F-HN) and F-HN co-administered with cholera toxin (CT, 1 μg) were tested in parallel. The vaccine was given on days 0 and 7, 3 μg/dose (10 μl per nare), and the responses were determined 4-6 weeks after the second vaccine dose.

As shown in **Table 2**, the free antigen, as well as the neutral and anionic Lip-HN were virtually ineffective mucosal vaccines. In contrast, the cationic Lip-HN, particularly those designated CAT4, CAT5 and CAT6 (the latter comprising of LLC), evoked a robust systemic and mucosal humoral response, with high levels of IgG1, IgG2a and IgA antibodies, namely a mixed Th1+Th2 response. No IgE antibodies were defected. The cationic liposomal vaccines CAT4-CAT6 also induced high levels of IFNγ (but not IL-4) in antigen-stimulated spleen cells. The responses produced by CAT6 were even stronger than those induced by F-HN adjuvanted with CT. Based on these findings, only the cationic formulations CAT4, 5 and 6 were further used.

The effect of the lipid/antigen w/w ratio and of liposomal cholesterol (Chol) on the immunogenicity of HN-loaded cationic liposomes

As mentioned above (Table 1) it was found that: (a) encapsulation efficiency of HN is markedly reduced at lipid/HN w/w ratios below 30/1, and (b) the addition of Chol to the formulations at cationic lipid/Chol mole ratios of 1/1 to 4/1 hardly affects the encapsulation efficiency. Thus the effect of these parameters on the immunogenicity of CAT4, 5 and 6 formulations was further tested. In addition, the immunogenicity of preformed liposomes simply mixed with the soluble antigen was determined. The data shown in **Table 3** indicate that all three formulations induce a strong systemic (serum) and local (lung) response, and that lowering the lipid/HN w/w ratio below 100/1 markedly reduces the response. The superiority of CAT6 (the cationic lipid of the present invention) over the other vaccine formulations is again seen as reflected by the high levels of serum and lung IgG2a and IgA antibodies (groups 12-16). Interestingly, simple mixing of soluble antigen

with preformed liposomes generated very potent vaccines (groups 18-20) that are equal to, or even more effective than, liposomes encapsulating the antigen. This suggests that real encapsulation of the antigen may not be necessary for the adjuvanticity of the cationic liposomes. Table 4 shows the results of the effect of Chol in the cationic liposome composition on the immune response. The addition of Chol slightly reduced the systemic HI response to CAT4 at 2/1 and 4/1 mole ratios (groups 4, 5), but not at a 1/1 mole ratio (group 3), and moderately enhances the overall response to CAT5 at all ratios (groups 7-9) and the local (lung) response to CAT6 at a 1/1 ratio (group 11).

Effect of the instillation volume on immunogenicity

After determining the optimal cationic lipid/antigen w/w ratio and the effect of Chol in the liposomal formulation (Tables 3, 4), the influence of the instillation volume. Lip (DOTAP/Chol, 1/1 mole ratio)-HN (CAT5), 3μg/dose, was administered twice i.n. in a total volume of 10, 20, 50 and 100 μl/dose (5, 10, 25 and 50 μl, respectively, per nare) and the response was assessed as described above. As can be seen in Table 5, the highest humoral response was obtained with 20 μl per dose (10 μl/nare). In view of the results presented in Tables 3-5, the following were defined as optimal for i.n. vaccination: (a) lipid/HN w/w ratio of 100-300/1, (b) cationic lipid/Chol mole ratio of 1/1, and (c) instillation volume 10 μl per nare.

The effect of adjuvant (CpG-ODN) on the immunogenicity of HN-loaded cationic liposomes

In the experiment presented in Table 6, an attempt was made to further enhance the response induced by HN-loaded cationic liposomes by the addition of free or liposome-encapsulated CpG-ODN (ODN 1018), shown by present inventors and others (data not shown) to be a potent mucosal adjuvant. In this experiment,

free or liposomal (CAT5) HN (2μg/dose) was co-administered i.n. with free or liposomal CpG-ODN (10 μg/dose). One group was immunized with HN+CpG-ODN co-encapsulated within the same liposomes. The CpG-ODN was encapsulated in either anionic (DMPC/DMPG, 9/1 mole ratio) or cationic (DMTAP/Chol, 1/1 mole ratio) liposomes.

The co-administration of free CpG-ODN with F-HN resulted in only a modest enhanced response (group 2). CpG-ODN entrapped in DMPC/DMPG anionic liposomes was 3-10 times (depending on the parameter tested) more efficacious as an adjuvant than free CpG-ODN (group 3) (data not shown). In comparison, CpG-ODN in DMTAP/Chol cationic liposomes was 7-60 times more potent than free CpG-ODN (group 4), indicating that the cationic liposomes are also a very efficient delivery system for the adjuvant CpG-ODN. However, addition of free or liposomal CpG to HN-loaded DMTAP/Chol liposomes had no beneficial immunostimulatory effect and, in some cases, even decreased the response (groups 6-8 vs. group 5). The only advantage of co-delivery of CpG-ODN+HN in the same liposomes (group 8) over the other formulations was the higher levels of serum and lung IgG2a antibodies.

These findings suggest that the addition of an exogenous mucosal adjuvant may not be necessary when using potent cationic liposomes, such as CAT4, 5 and 6 (the present invention) for i.n. antigen delivery.

Induction of cellular responses

Mice were immunized i.n. with various cationic liposomal formulations and the splenocyte cellular responses — cytotoxicity, proliferation and IFNγ production – were measured 6 weeks after vaccination. In the experiment shown in Table 7, a comparison was made between HN-loaded liposomes (groups 3-10) and free antigen (F-HN) admixed with preformed empty liposomes (groups 11-13). We also compared the immunogenicity of CAT5-HN and CAT6-HN (the present invention) prepared at varying lipid/HN w/w ratios (30/1-300/1).

Preferential cytotoxicity against the specific target cells (P815 pulsed with the influenza peptide) was obtained only with CAT6-HN at a lipid/HN w/w ratio of 100/1 (group 8) and with all the three preformed liposomes (CAT4, 5, 6) co-administered with free antigen. The maximum proliferative response was observed with CAT5-HN at lipid/HN w/w ratios of 50/1 and 30/1 and with CAT6-HN at 300/1, 100/1 and 50/1 ratios. The proliferative and cytotoxic responses elicited by the most efficacious liposomal formulations were 2-3 times greater than those induced by free antigen.

These findings suggest that as compared with the humoral response (Table 3), where the highest levels of all types of antibodies measured were obtained at lipid/HN w/w ratios of 100/1-300/1, lower w/w ratios (e.g. 30/1-100/1) may be optimal for the cellular responses. Moreover, whereas CAT5-HN elicits a strong humoral response, this formulation is a poor inducer of cytotoxic activity, as compared with CAT6-HN. Interestingly, vaccination with mixtures of free antigen with preformed cationic liposomes (all three formulations) in suspension evokes good cellular responses that are similar in magnitude to those induced by the encapsulated antigen. Thus, simple mixing of free antigen with preformed cationic liposomes may be sufficient to induce both strong humoral (Table 3) and cellular (Table 7) responses. Further studies comparing the efficacy of encapsulated antigen and antigen-cationic liposome mixtures are warranted.

Effect of the route of vaccine administration on humoral and cellular responses and protective immunity

In the experiment shown in Table 8, a comparison was made between 1 i.m. dose, 1 or 2 i.n. doses and 2 oral doses of HN-loaded cationic liposomes (CAT4, 5, 6 the lattrer comprising the LLC of the present invention) with regard to immunogenicity and induction of protective immunity to live virus challenge. Of the various routes, i.n. administration X2 generates the strongest humoral and cellular response and protective immunity. Of the 3 formulations, CAT 6 (the

present invention) induces the highest response, particularly with regards to serum and lung IgG2a and IgA antibodies. Other experiments demonstrated that Cat 4, 5 and 6 (LLC) induce high levels of MHCII, CD40 and B7 molecules in mouse peritoneal macrophages following 48h incubation. CAT6 produces the highest levels. Since these molecules are essential in antigen presentation, these changes in surface molecules of antigen – presenting cells can explain, at least in part, the superior immunostimulatory activity of CAT6.

In Conclusion: Of the 8 different liposomal formulations tested (a neutral, an anionic and six cationic), CAT 6 (the present invention) proves to be the most efficient delivery system for the influenza antigens. This formulation is even more potent than CT, which is regarded as the most powerful mucosal adjuvant, yet toxic and not approved for human use.

EXAMPLES

Chemistry

Example 1: monosperminecerami**d**e-1-O-[N,N-3-aminopropyl-4-(3 amino-propylamino)butyl]carbamoyl–2-hexadecanoylamino–4–octadecene–1,3-diol

(i) N-palmitoylsphingosine (1.61g, 3mmol) was dissolved in dry THF (100ml) with heating. The clear solution was brought to room temperature and N,N'-disuccinimidyl carbonate (1.92g, 7.5 mmol) was added. DMAP (0.81g, 7.5 mmol) was added with stirring and the reaction further stirred for 16 hours. The solvent was removed under reduced pressure and the residue recrystallized from n-heptane yielding 1.3g (68%) of disuccinimidylceramidyl carbonate as white powder m.p. 73-76°C.

(ii) Spermine (0.5g, 2.5 mmol) and disuccinimidylceramidyl carbonate (0.39g, 0.5 mmol) were dissolved in dry dichloromethane with stirring and then treated with catalytic amount of DMAP. The solution was stirred at room

temperature for 16 hours, the solvent evaporated and the residue treated with water, filtered and dried in vacuo, giving 0.4g (82%) of crude material which was further purified by column chromatography on Silica gel, using 60:20:20 Butanol: AcOH:H_2O eluent.

(iii) Methylation of the compound obtained in (ii) with DMS or CH_3I.

Example 2:Preparation of Liposomes

Liposomes were prepared by dissolving the appropriate cationic lipid (DOTAP-1,2-dioleoyl-3-trimethylammonium-propane, DC-CHOL-3β[N-(N',N'–dimethyl-aminoethane)carbamoyl]cholesterol or the product of Example 1) or cationic lipid/neutral co-lipid (DOPC-1,2-dioleoyl-*sn*-glycero-3 phosphatidyl-choline, DOPE-dioleoylphosphatidylethanol-amine or cholesterol) mixture (1/1 mole ratio) in tert-butanol. The mixture was centrifuged under vacuum for 3 hours in an Automatic Environmental Speedvac, Model AE2010 (Savant Instruments Inc, Holbrook, NY) such that all the tert-butanol had evaporated. The powder thus formed was hydrated with 20mM hepes buffer (pH 7.4) to produce a heterogeneous vesicle (HV) dispersion exhibiting a total lipid concentration of 31mM. If required, these HVs, exhibiting ~500–1500 nm diameter, could be downsized and converted to large unilamellar vesicles (LUVs), exhibiting ~100 nm diameter, by the use of a 'Liposofast' extrusion system (MacDonald et al. 1991) as previously described (Meidan et al. 2000). In all cases, the prepared liposomes were stored at 4°C. Just before their addition to cells, small volumes of liposome dispersion were diluted with pre-calculated amounts of 20mM Hepes buffer (pH 7.4) in order to produce aliquots containing the appropriate amount of cationic lipid.

Example 3: Preparation of Lipoplexes containing oligonulcleotides

For the preparation of the lipoplexes, the diluted liposome dispersions (either HV or LUV) obtained in Example 2 were added to solutions comprising an 18-mer antisense phosphorothioate oligonucleaotide (S-ODN), appropriately diluted in 20mM Hepes buffer at pH 7.4, in glass vials at the appropriate

L^+/ODNP mole ratio. It should be noted that each cationic lipid molecule in the compound formed in Example 1 exhibits an equivalent of two positive charges and this was taken into consideration for calculating L+. In all cases, lipoplexes were formed by allowing the mixture to stand for 20 to 40 min with occasional mild vortexing (Meidan et al,).

Example 4: Lipofection

150µl Aliquots of culture medium (incorporating 10% v/v fetal calf serum), each containing 4 x 10^3 cells, were deposited into the wells of a flat bottom 96 well plate (Corning Inc, NY). A 50µl volume of the appropriate treatment solution was then added to each seeded well. The treatment solutions contained S-ODN (generally 0.005 nmol unless otherwise stated), either free or as freshly prepared lipoplexes; or alternatively control liposome solutions containing equivalent amounts of cationic lipid. In all cases, the cells were incubated for 96 h at 37°C, 90% relative humidity and 5% CO_2. Each specific treatment solution was added to three different wells and the entire experiment was performed in triplicate, each time using a different batch of cultivated cells. *i.e.* each treatment solution was added to 9 wells together.

Example 5: Encapsulation of antigens in liposomes

The HN antigens {influenza A/New Caledonia/20/99-like (H1N1)} were encapsulated in large (mean diameter 0.5-1 µm) heterogeneous vesicles (HV) consisting of either: (a) dimyristoyl-phosphatidylcholine (DMPC, neutral liposomes); (b) DMPC and dimyristoyl-phosphatidylglycerol (DMPG) (both from Lipoid GmbH, Ludwigshafen, Germany) at a DMPC:DMPG mole ratio of 9:1 (anionic liposomes); or (c) cationic (CAT) lipids, alone and with cholesterol (Chol) at 1:1 to 4:1 mole ratio, respectively, or with the neutral lipid dioleoyl-phosphatidylethanolamine (DOPE) at a 1:1 mole ratio (cationic liposomes). The following commercial cationic lipids (a-e) were used:

(a) Dimethylaminoethane-carbamol-cholesterol (DC-Chol:DOPE) (CAT1),

(b)Distearoyl-trimethylammonium-propane-cholesterol (DSTAP) (CAT2),

(c) Dimethyldioctadecylammonium-bromide-cholesterol (DDAB) (CAT3),

(d) Dioleoyl-trimethylammonium-propane-cholesterol (DOTAP) (CAT4),

(e) Dimyristoyl-trimethylammonium-propane-cholesterol (DMTAP) (CAT5) and

(f) The lipid-like cationic (LLC) vehicle of the present invention-cholesterol (CAT6) as synthesized by the inventors.

For all formulations, the following procedure was used. Lipids (10-30 mg) were dissolved in 1ml tertiary butanol, then sterilized by filtration (GF92, Glasforser, Vorfilter no. 421051, Schleicher & Schuell, Dassel, Germany). The sterile lipid solution was frozen at -70°C, then lyophilized for 24 h to complete dryness. The dried lipids could be stored at 4°C for >1 year without significant (<10%) lipid degradation or loss or encapsulation capability. Upon need, the lipid powder was hydrated with the antigen solution (in saline) at a lipid:antigen w/w ratio of 3:1 to 300:1. The antigen solution was added stepwise in increments of 30-50 µl and vortexed vigorously after each addition, up to a final volume of 0.5-1 ml. In some experiments, the antigen solution was mixed, using vortex, with preformed empty liposomes suspended in saline or PBS.

To determine encapsulation efficiency, liposomes were centrifuged under conditions where ≥90% of the liposomes precipitate while ≥80% of the non-encapsulated antigen remains in the supernatant. Non-cationic liposomes containing the influenza HN antigens were centrifuged at 4°C for 30 min at 14,000 rpm and the amount of non-encapsulated protein was determined by the modified Lowry technique. For the cationic liposomes, a sample (0.5 ml) was loaded very carefully using a gel loading tip (100-150 µl at a time) over 0.5 ml of D_2O, then centrifuged for 1 h at 30°C at 45,000 rpm. This results in the free, non-encapsulated HN precipitating into a pellet at the bottom of the eppendorf tube, while the liposomal HN and a few empty liposomes float at the top of, or in the supernatant. To determine encapsulation, the entire supernatant was collected

with a Pasteur pipette. The liposomes and the pellet were then dissolved with 200 µl of warm 10% Triton X–100 added to each fraction. The Lowry method (modified to 20% SDS procedure) was used to determine protein concentration in each fraction.

Example 6: Immunization

Free (F-HN) and liposomal (Lip-HN) vaccines, 1-4 µg/dose, were administered either once intramuscularily (i.m., in 30 µl), once or twice intranasally (i.n., in 5-50 µl per nare), or twice orally (in 50 µl), spaced 1 week apart. In all cases, mice were lightly anesthetized with 0.1 ml of 4% chloral hydrate in PBS given intraperitoneally. For oral administration mice were treated with an antiacid 30 min prior to vaccination. Cholera toxin (CT, Sigma, USA), 1 µg/dose, was used in all experiments as a standard mucosal adjuvant for comparison. In one experiment, CpG-ODN (ODN 1018 generously provided by Dr. E. Raz, University of California, San Diego, CA, USA), 10 µg/dose, was used as an adjuvant.

Example 7: Assessment of humoral responses

Sera, lung homogenates and nasal washes, prepared as described previously, were tested, individually or pooled, 4-6 weeks post-vaccination, starting at 1/10 or 1/20 sample dilution. Hemagglutination inhibiting antibodies were determined by the standard hemagglutination inhibition (HI) assay. Mice with HI titer $\geq$40 (considered a protective titer in humans) were defined as seroconverted. Antigen-specific IgG1, IgG2a, IgA and IgE levels were measured by ELISA. The highest sample dilution yielding absorbance of 0.2 OD above the control (antigen + normal mouse serum, OD <0.1) was considered the ELISA Ab titer.

Example 8: Assessment of cellular responses

Splenocytes obtained at 4-6 weeks after vaccination were tested for proliferative response, IFNγ and IL-4 production, and cytotoxic activity, following

in vitro stimulation with the antigen. Cultures were carried out at 37°C in enriched RPMI 1640 or DMEM medium supplemented with 5% (for proliferation, cytokines) or 10% (for cytotoxicity) FCS, with (for cytotoxicity) or without 5 x 10^{-5}M 2-mercaptoethanol. Cell cultures were performed as follows, (i) Proliferation: 0.5 x 10^6 cells per well were incubated in U-shaped 96-well plates, in triplicate, with or without the antigen (0.5-5 µg per well), in a final volume of 0.2 ml. After 72-96h. cultures were pulsed with 1 µCi ^{3}H-thymidine for 16 h. Results are expressed in Δcpm = (mean counts per minute of cells cultured with antigen) – (mean counts per minute of cells cultured without antigen). (ii) Cytokiines: 2.5 x 10^6 to 5 x 10^6 cells per well were incubated in 24-well plates, in duplicate, with or without the antigen (5-10 µg per well), in a final volume of 1 ml. Supernatatants were collected after 48-72 h and tested by ELISA for murine IFNγ and IL-4 using the Opt EIA Set (Pharmingen, USA). (iii) Cytotoxicity: Responding splenocytes ($2.5x10^6$) were incubated as in (ii) for 7 days together with an equal number of stimulating BALB/c splenocytes that had been infected with the X/127(H1N1) influenza virus (see below). For infection, the splenocytes were incubated, with occasional stirring, for 3 h at 37°C in RPMI 1640 medium (without FCS) with 150 hemagglutination units/$1x10^6$ splenocytes of the virus, followed by washing. Subsequently, the primed effector cells were restimulated for 5 days with infected, irradiated (3,000 rad) splenocytes at an effector/stimulator cell ratio of 1/4 in the presence of 10 IU/ml of rhIL-2. Cytotoxicity was measured using the standard 4 h ^{51}Cr release assay at effector/target cell ratios of 20/1 and 100/1. The labeled target cells used were unmodified P815 and P815 pulsed for 90 min at 37°C with the HA2 189-199 peptide (IYSTVASSLVL, 20 µg/$1x10^6$ cells).

Example 9: Determination of protective immunity

Mice were anesthetized and 25 µl of live virus suspension per nostril was administered, using the reassortant virus X-127 (A/Beijing/262/95 (H1N1) x X-31 (A/Hong Kong/1/68 x A/PR/8/34), which is infectious to mice and cross-reactive

with A/New Caledonia, 10^7 EID 50 (egg-infectious dose 50%). The lungs were removed on day 4, washed thrice in cold PBS, and homogenized in PBS (1.5 ml per lungs per mouse, referred to as 1/10 dilution). Hemogenates of each group were pooled and centrifuged at 2000 rpm for 30 min at 4°C and the supernatants collected. Serial 10-fold dilutions were performed and 0.2 ml of each dilution was injected, in duplicate, into the allantoic sac of 11-day-old embryonated chicken eggs. After 48 h at 37°C and 16 h at 4°C, 0.1 ml of allantoic fluid was removed and checked for viral presence by hemagglutination (30 min at room temperature) with chicken erythrocytes (0.5 wt.%, 0.1 ml). The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination).

Statistical analysis

Differences in HI antibody titer between groups were analyzed using a two-tailed Student's *t*-test. *P*-values <0.05 sere considered significant.

MATERIALS AND METHODS

Materials

S-ODN: Phosphorothioate antisense 18-mer G3139 [S-d-5'-(TCT CCC AGC GTG CGC CAT)] was obtained from Genta Inc. Lexington, MA. Its purity was assessed by HPLC (Merck Hitachi D-7000) incorporating an anion exchange column. A gradient of ammonium acetate in 50% isopropanol in a pH 8 buffer was employed. For each ODN, a single sharp peak was obtained, thus confirming that our supplies of both G3139 and G3622 were indeed pure. The ODNs were dissolved in 20 mM Hepes buffer (pH 7.4) and ODN concentrations were determined by optical density measurements (Shimadzu UV-24101PC, Duisburg, Germany) made at 254 nm (Brown T, Brown DJS, 1991. Modern machine-aided methods of oligonucleotide synthesis, In: Eckstein F editor. Oligonucleotides and Analogues, A Practical Approach. New York: Oxford University Press, pp. 20). By multiplying the derived

ODN concentration values by 17, it was possible to obtain the corresponding phosphate anion ($ODNP^-$) concentration. The ODN phosphate concentration was confirmed by a direct organic phosphorus determination.

Cell culture

MCF-7 cells were cultivated in DMEM supplemented with 2mM L-glutamine, 100 IU/ml penicillin-streptomycin, 4.5g/L D-glucose, 1 mM sodium pyruvate and 10% v/v fetal calf serum. For the lipoplex studies, the MCF-7 cells were cultivated as above with the exception that no pyruvate was incorporated. The cells were grown to ~90% confluency in 75cm^2 flasks. All the cell culture medium components were acquired from Biological Industries (Kibbutz Beit Haemek, Israel).

Western Blot

Cell extracts were obtained by lysing the cells in protein lysis buffer (Tris-HCl pH 6.8, 2.24% glycerol, 6% SDS, 0.02% bromophenol blue, 10% β-mercaptoethanol) for 10 min at 100°C and subsequent centrifugation. The extracts were subjected to polyacrylmide gel electrophoresis (PAGE-SDS). Bradford dye assay (Bio Rad Laboratories, Hercules, CA) indicated that each lane contained 50μg of protein. Anti human bcl-2 mAb (100) (Santa Cruz Biotechnolgy, Inc., Santa Cruz, CA) was used. Detection was performed by employing horse radish peroxidase conjugated to goat anti-mouse IgG (H+L) (Jackson Immuno Research Lab. Inc., West Grove, PA), followed by ECL (Amersham, Pharmacia Biotech, Uppsala, Sweden). Antibodies were stripped from the membrane by 1% SDS in phosphate-buffered saline. The detection of actin levels by the use of anti-human actin mAb (Roche Molecular Biochemicals, Annheim, Germany) served as an internal control.

Luciferase assay

Luciferase activity was assayed on cell extracts using Bright-Glo™ Luciferase Assay system (Promega, Madison WI). Briefly, the cellwere washed 2 times with cold PBS x 1 and lysed for 10 min with Cell Culture Lysis Buffer (Promega). Then, cells were harvested and centrifuged for 5 min to remove cell debris. The amount of enzyme was measured on Terner luminometer (Promega) quantified in supernatants and was quantified by generating calibration curve using recombinant enzyme QuantiLum® (Promega, Madison WI). The amount of luciferase was normalized to the amount of total protein in the lysate, which was determined by Lowry method.

Mice

Specific pathogen-free (SPF) female BALB/c mice, 6-8 weeks old, were used (5-10 per group). Animals were maintained under SPF conditions.

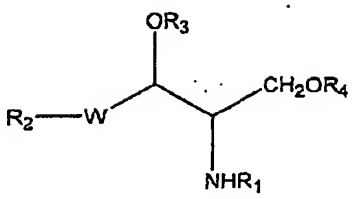
Influenza antigens

A monovalent subunit antigen preparation derived from influenza A/New Caledonia/20/99-like (H1N1) strain was generously provided by Drs. Gluck and Zurbriggen, Berna Biotech, Bern, Switzerland. This preparation (designated herein HN) comprised of 80-90% hemagglutinin, 5-10 wt.% neuraminidase and trace amounts of NP and M1 proteins. A whole-inactivated virus was used in some experiments for in vitro stimulation.

The invention will now be defined by the appended claims, the contents of which are to be read as included within the disclosure of the specification.

CLAIMS:

1. A compound of formula (I):



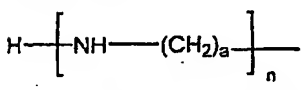
wherein

R_1 is selected from hydrogen, branched or normal alkyl, aryl, lower alkyl amine wherein said amine may be substituted, or $C(O)R_5$;

W is $-CH=CH-$, $-CH_2-CH(OH)-$ or $-CH_2-CH_2-$,

R_2 and R_5 are independently selected from a saturated or unsaturated normal or branched C_{10}-C_{24} alkyl, alkenyl or polyenyl groups;

R_3 and R_4 are independently selected from hydrogen, a group $-C(O)-NR_6X-Z$, Z being a same or different for R_3 and R_4; or R_3 and R_4 form together with the oxygen atoms to which they are bound a heterocyclic group comprising $-C(O)-NH-Z-NH-C(O)-$; R_6 is hydrogen or

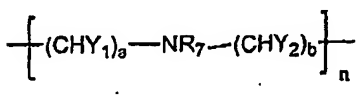


wherein a represents an integer from 1 to 4; n represents an integer from 1 to 6;

Z being a branched or linear polyalkylamine chain wherein said polyalkylamine chain may comprise quaternary ammonium groups;

with the proviso that R_3 and R_4 are not simultaneously hydrogen.

2. The compound of Claim 1, wherein said polyalkylamine chain has the general formula (II):

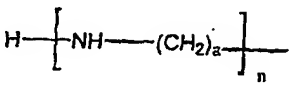


wherein

a and b are independently selected from 1, 2, 3 or 4;

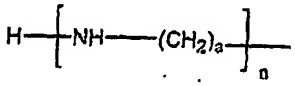
n represents an integer from 1 to 6;

R_7 is selected from hydrogen or



where a and n have the meaning as above; and Y_1 and Y_2 are independently selected from hydrogen and alkyl.

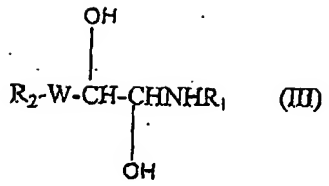
3. A compound according to claim 2 wherein R_1 is a $C(O)R_5$ group, W is –CH=CH-, R_2 and R_5 are independently selected from a saturated or unsaturated linear or branched C_{12}-C_{18} alkyl or alkenyl groups; R_3 and R_4 are independently selected from hydrogen and –C(O)-NR_6X-Z, R_6 is hydrogen or



and Z is a polyalkylamine chain.

4. A compound according to claim 2 wherein wherein R_1 is a $C(O)R_5$ group, W is –CH=CH-, R_2 and R_5 are independently selected from a saturated or unsaturated linear or branched C_{12}-C_{18} alkyl or alkenyl groups; R_3 and R_4 form together with the hydrogen atoms to which they are bonded a heterocyclic group comprising –C(O)-NH–Z–NH-C(O)–, Z being a branched or linear polyalkyl amine chain wherein said polyalkylamine chain may comprise quaternary ammonium groups.

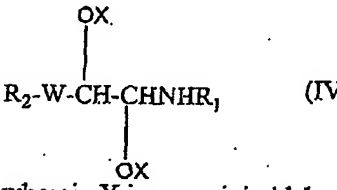
5. A process for the preparation of a compound of formula (I) comprising the steps of reacting a compound of formula (III)



(III)

wherein R_1, R_2 and W have the meaning as defined above;

with an activating compound selected from N,N'-disuccinimidyloxycarbonate, di- or tri-phosgene or an imidazole residue to form a compound of formula (IV)



(IV)

wherein X is a succinimidyloxycarbonyl, a chloroformate or an imidazole residue; said compound of formula (IV) being further reacted with one or two equivalents of branched or linear polyalkylamine to form a compound of formula (I).

6. A method for introducing biologically active compounds into eukaryotic cells comprising forming liposomes from the compound of formula (I), contacting said liposomes with a biologically active compound to form a complex and administering said complex.

7. A complex comprising liposomes formed from the compound of formula (I) and a biologically active compound.

8. A complex according to claim 7 wherein said biological active compounds are selected from the group comprising of genes, polynucleotides, oligonucleotides, proteins, antigens, peptides and drugs.

9. A pharmaceutical composition comprising a complex as claimed in claim 7 together with suitable excipients.

10. A pharmaceutical composition according to claim 9, which is administered intravenously, subcutaneously, topically, intranasally, orally, ocularly, intramuscularly.

11. A method of treatment of cancer in a subject comprising the administration a pharmaceutical composition as claimed in claim 9 to said subject.

12. A method according to claim 11, wherein said cancer is breast cancer.

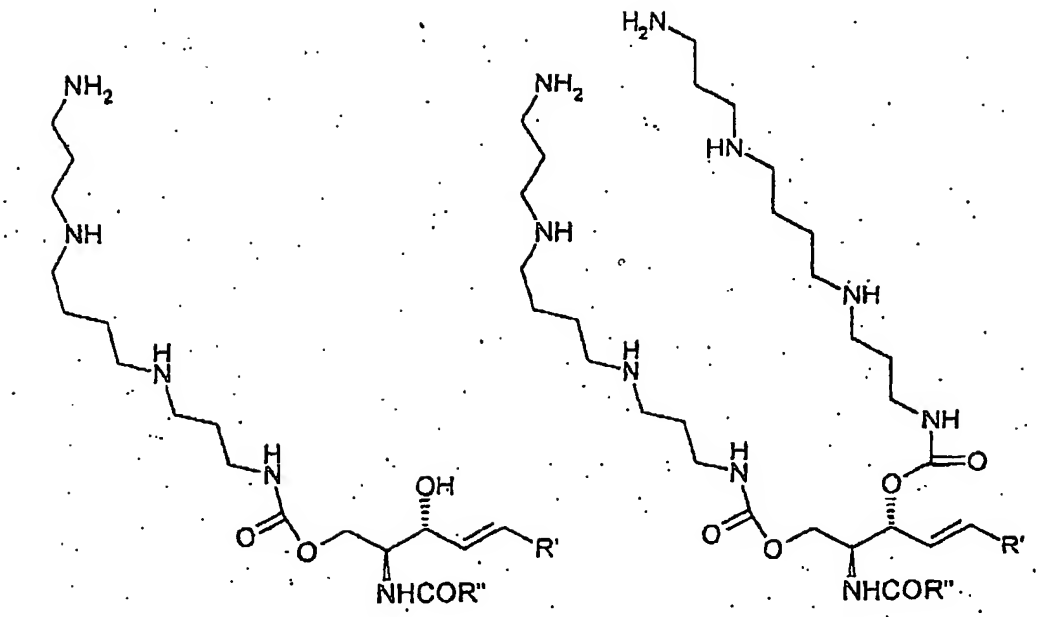
13. A method of vaccination of a subject in need thereof comprising the administration of a composition as claimed in claim 9.

14. A method according to claim 13 wherein said vaccination is done mucosally, preferably intranasally.

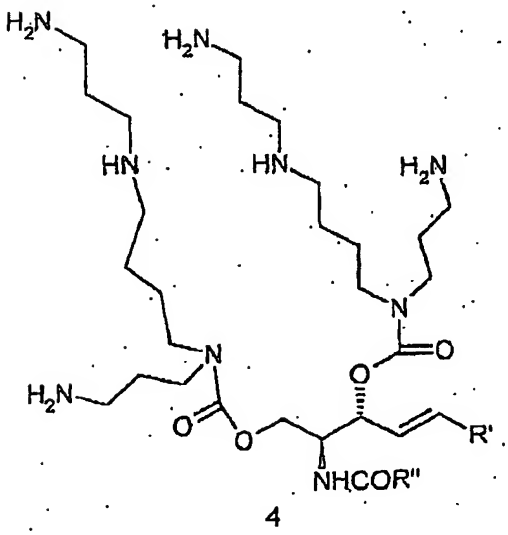
15. A method of transfection of a gene into cells comprising contacting a complex as claimed in claim 7 with cells.

Abstract

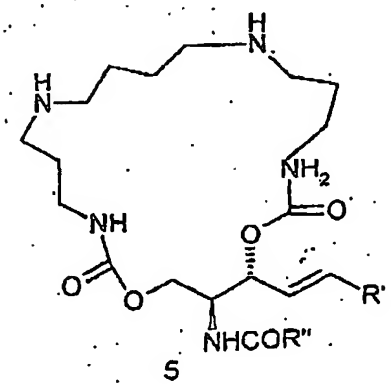
The present invention concerns compounds of formula (I) and their use as lipid-like vehicles for delivering active biological moieties into cells for therapy, immunization and gene transfer.



"linear" ceramide-Sprm conjugates



"branched" ceramide-Sprm conjugate



"cyclic" ceramide-Sprm conjugate

Fig. 1

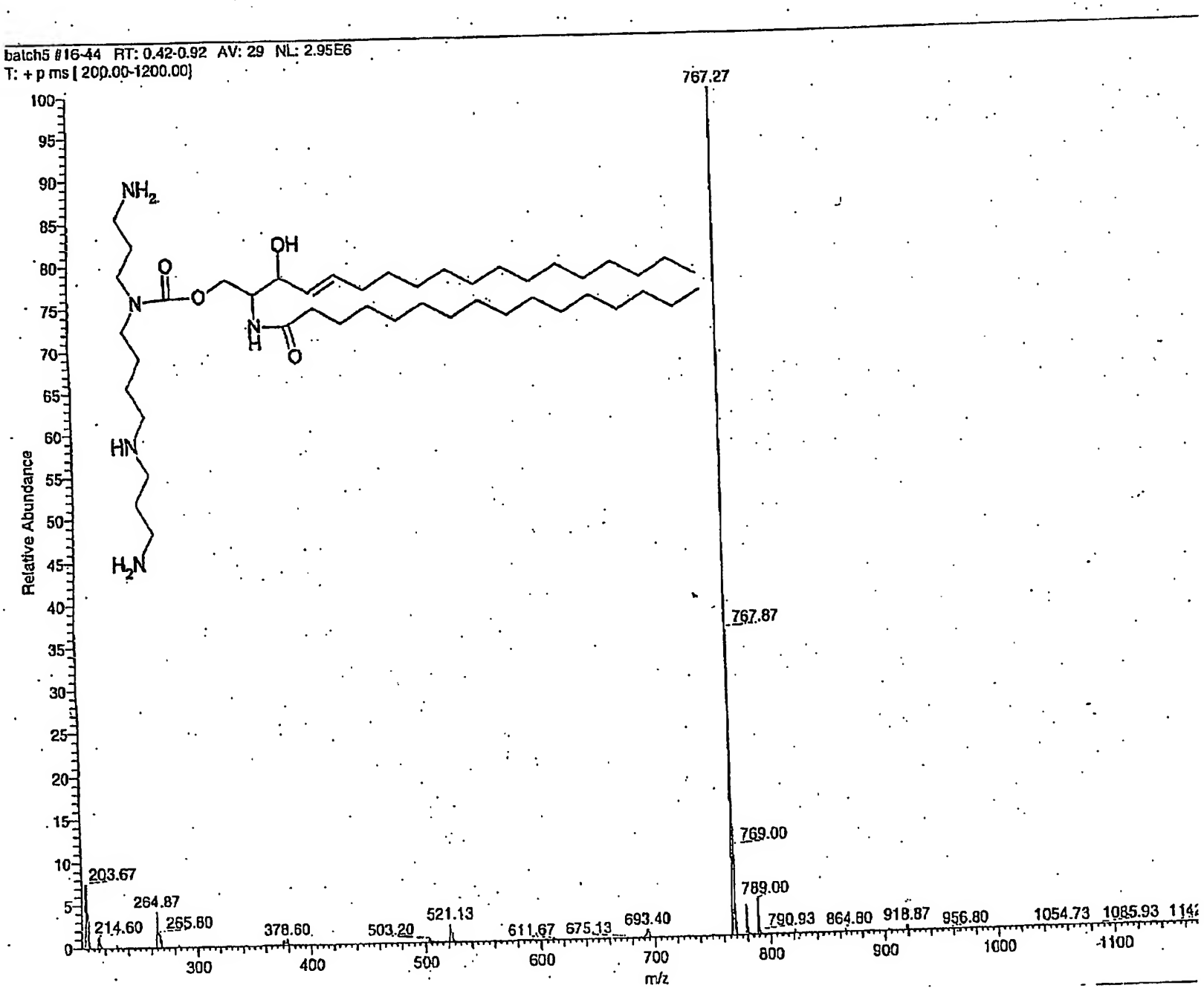

batch5 #16-44 RT: 0.42-0.92 AV: 29 NL: 2.95E6
T: + p ms [200.00-1200.00]
Relative Abundance
m/z
767.27
767.87
769.00
789.00
203.67
264.87
214.60
265.80
378.60
503.20
521.13
611.67
675.13
693.40
790.93
864.80
918.87
956.80
1054.73
1085.93
NH2
OH
O
N
O
NH
O
HN
H2N

Fig. 2

Fig. 6A

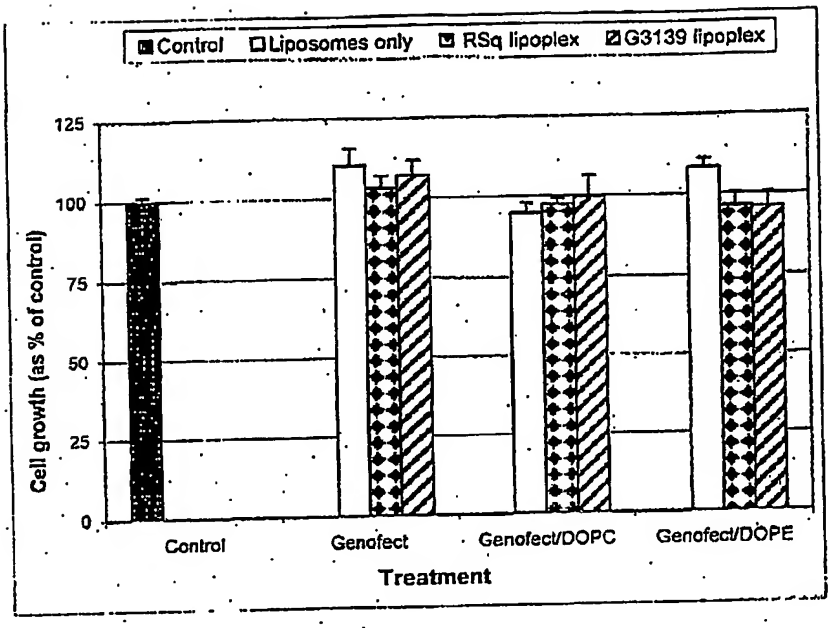


Fig. 6B

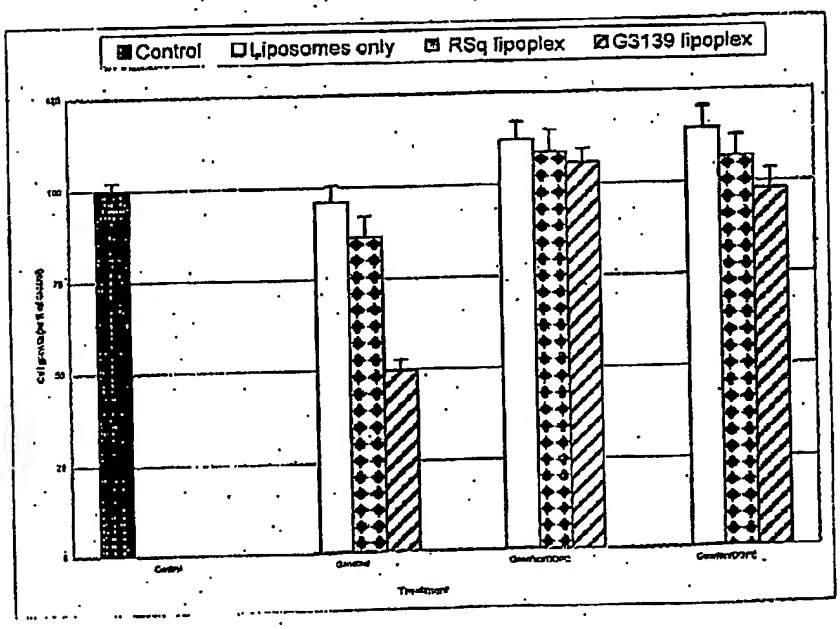


Fig. 3

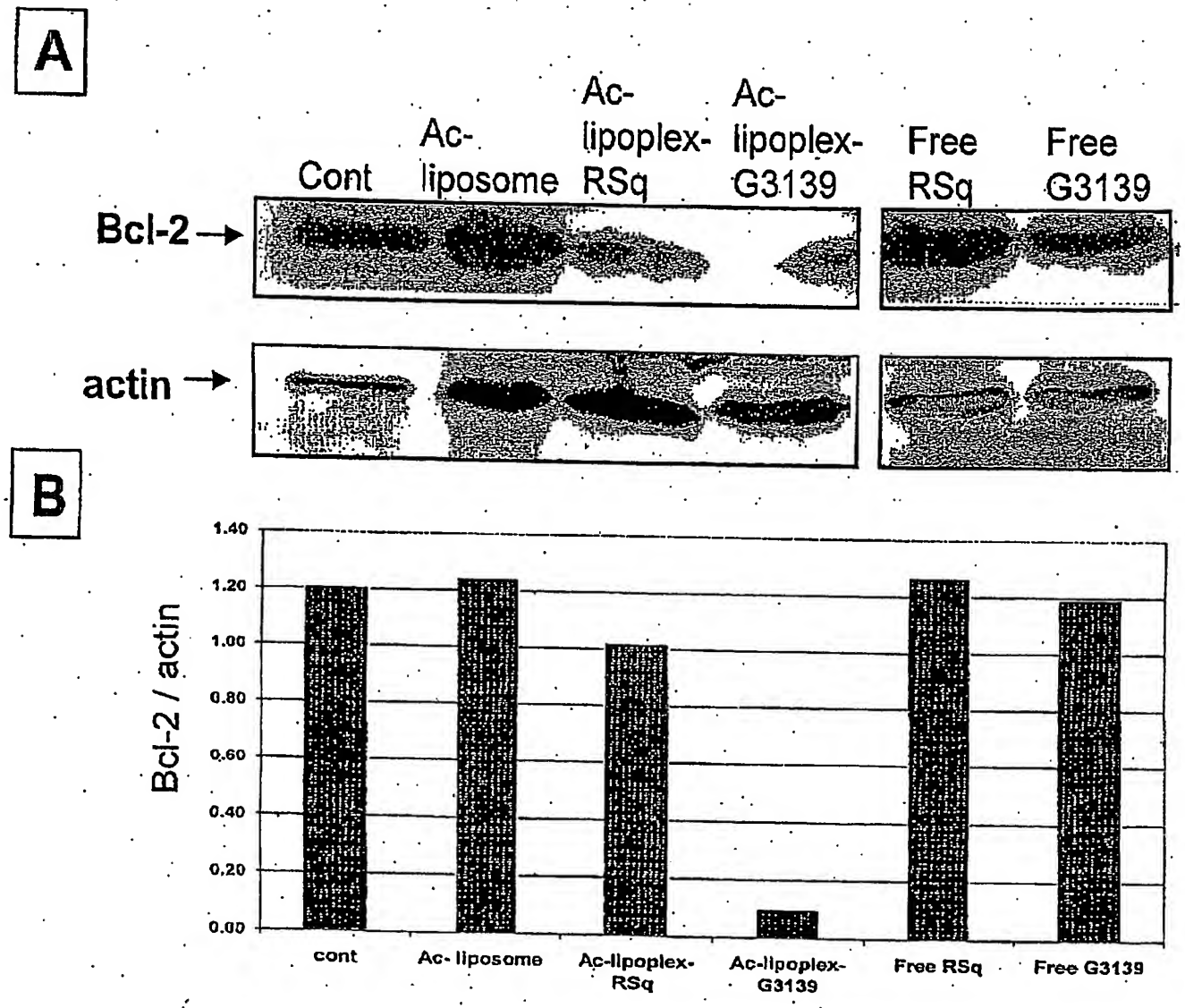

A
Cont
Ac-liposome
Ac-lipoplex-RSq
Ac-lipoplex-G3139
Free RSq
Free G3139
Bcl-2
actin
B
Bcl-2 / actin
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
cont
Ac- liposome
Ac-lipoplex-RSq
Ac-lipoplex-G3139
Free RSq
Free G3139

Fig. 4

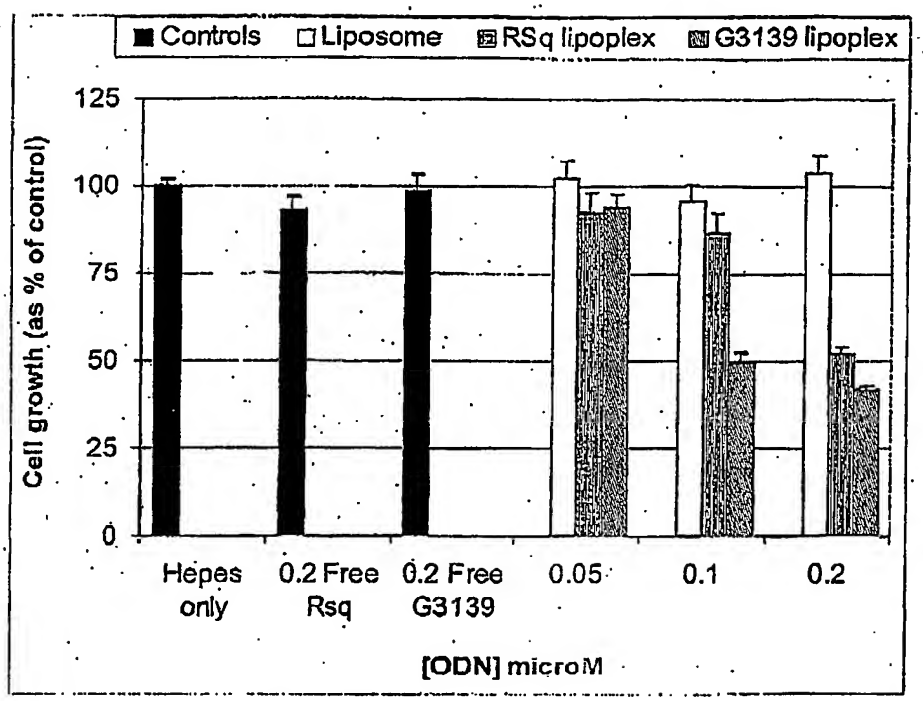

Controls
Liposome
RSq lipoplex
G3139 lipoplex
Cell growth (as % of control)
125
100
75
50
25
0
Hepes only
0.2 Free Rsq
0.2 Free G3139
0.05
0.1
0.2
[ODN] microM

Fig. 5

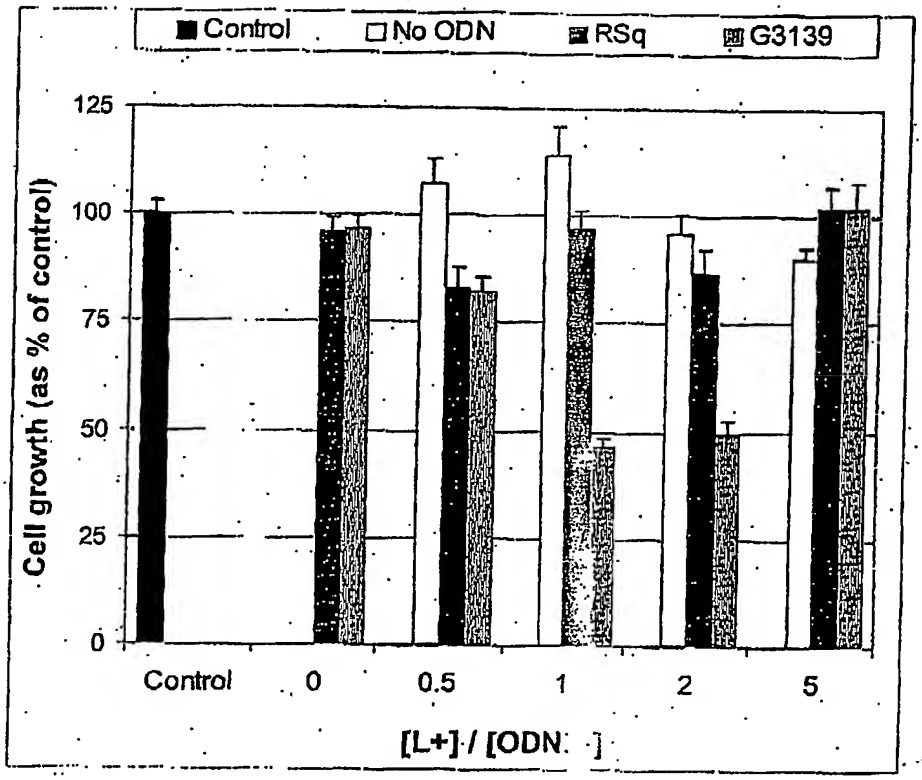

Control
No ODN
RSq
G3139
125
100
75
50
25
0
Cell growth (as % of control)
Control
0
0.5
1
2
5
[L+] / [ODN]

Fig. 6

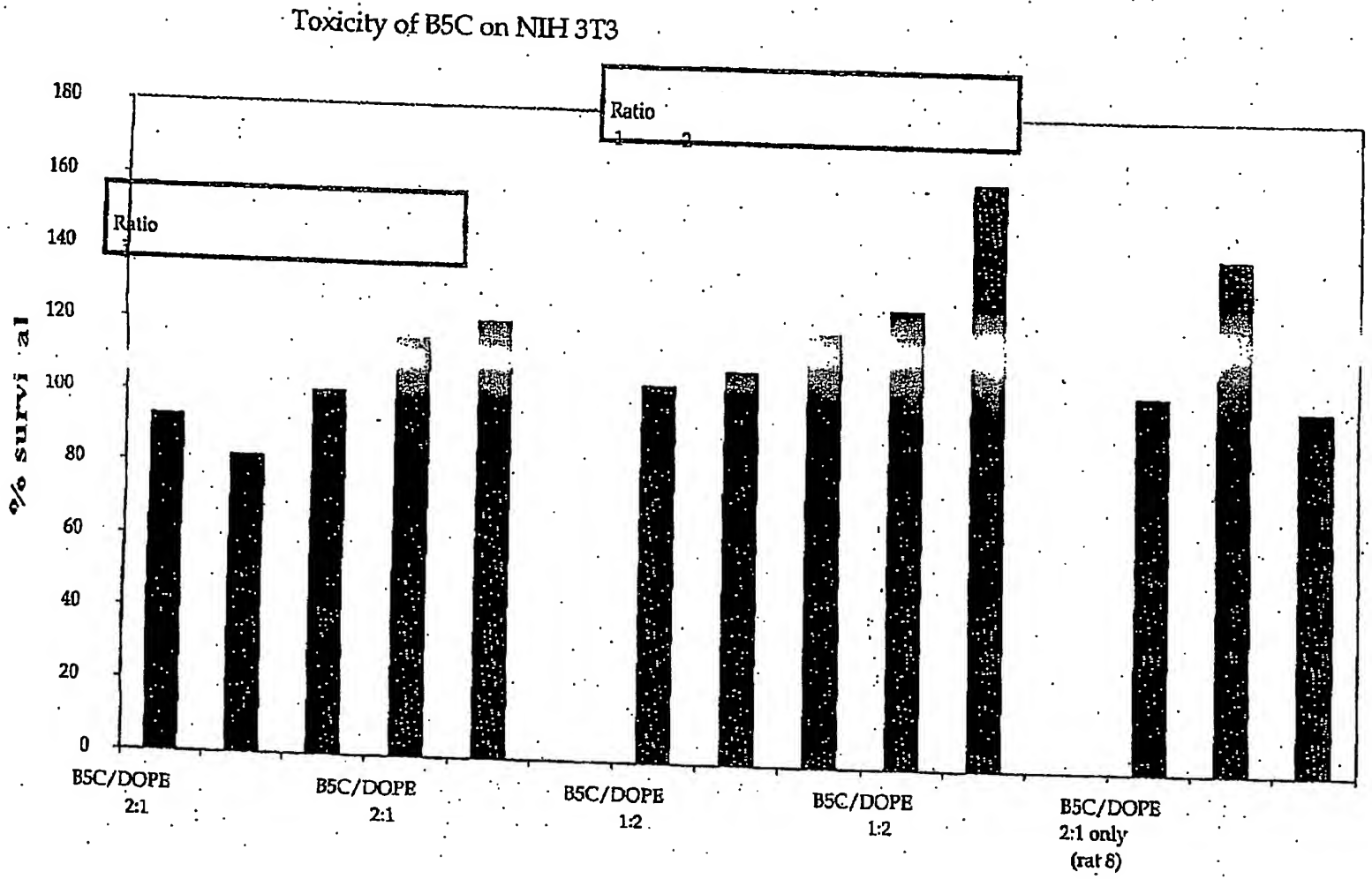

Toxicity of B5C on NIH 3T3
Ratio
1
2
Ratio
% survival
160
160
140
120
100
80
60
40
20
0
B5C/DOPE
2:1
B5C/DOPE
2:1
B5C/DOPE
1:2
B5C/DOPE
1:2
B5C/DOPE
2:1 only
(rat 8)

Fig. 7

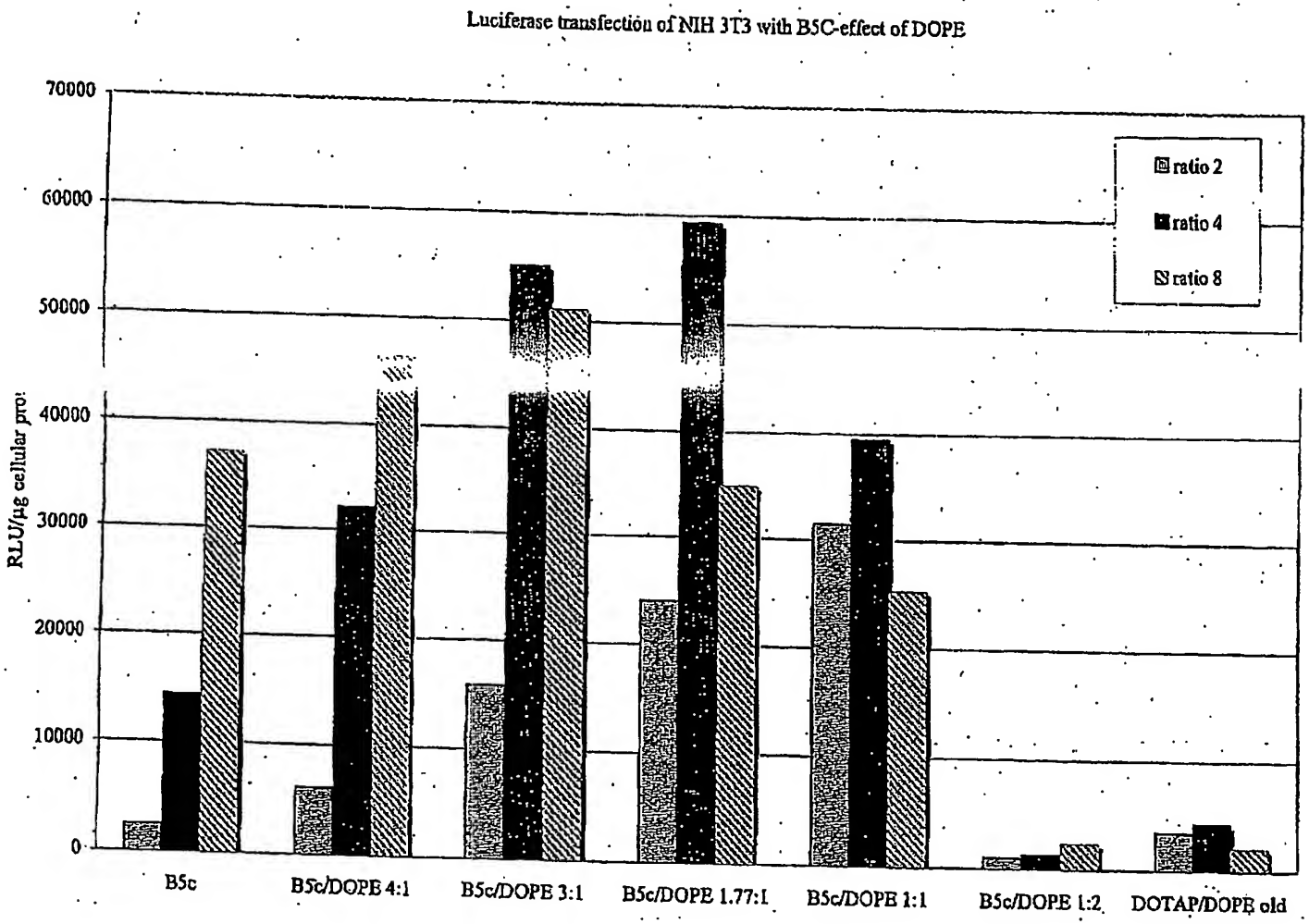

Luciferase transfection of NIH 3T3 with B5C-effect of DOPE
70000
60000
50000
40000
30000
20000
10000
0
RLU/µg cellular pro
ratio 2
ratio 4
ratio 8
B5c
B5c/DOPE 4:1
B5c/DOPE 3:1
B5c/DOPE 1.77:1
B5c/DOPE 1:1
B5c/DOPE 1:2
DOTAP/DOPE old

Fig. 8

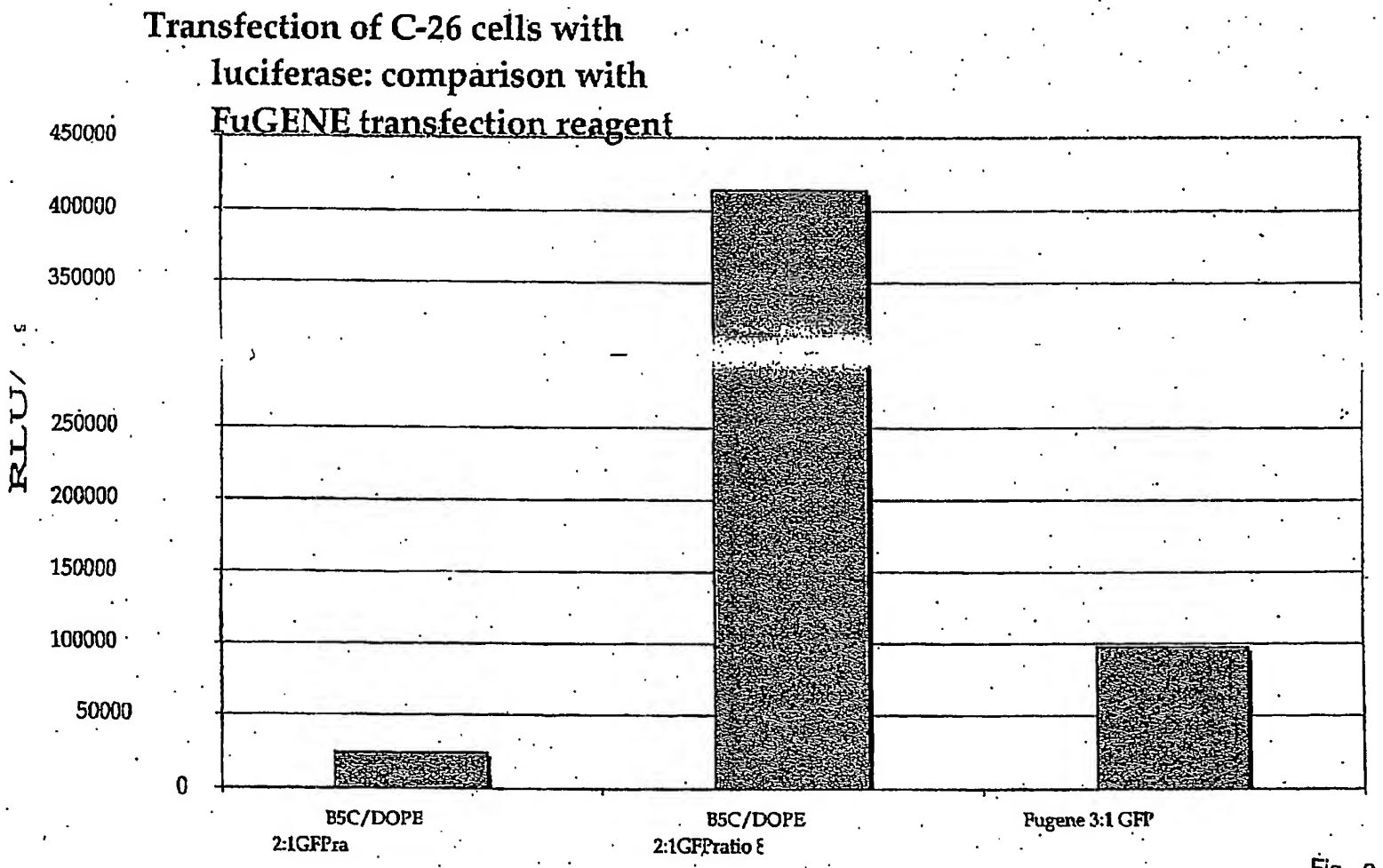
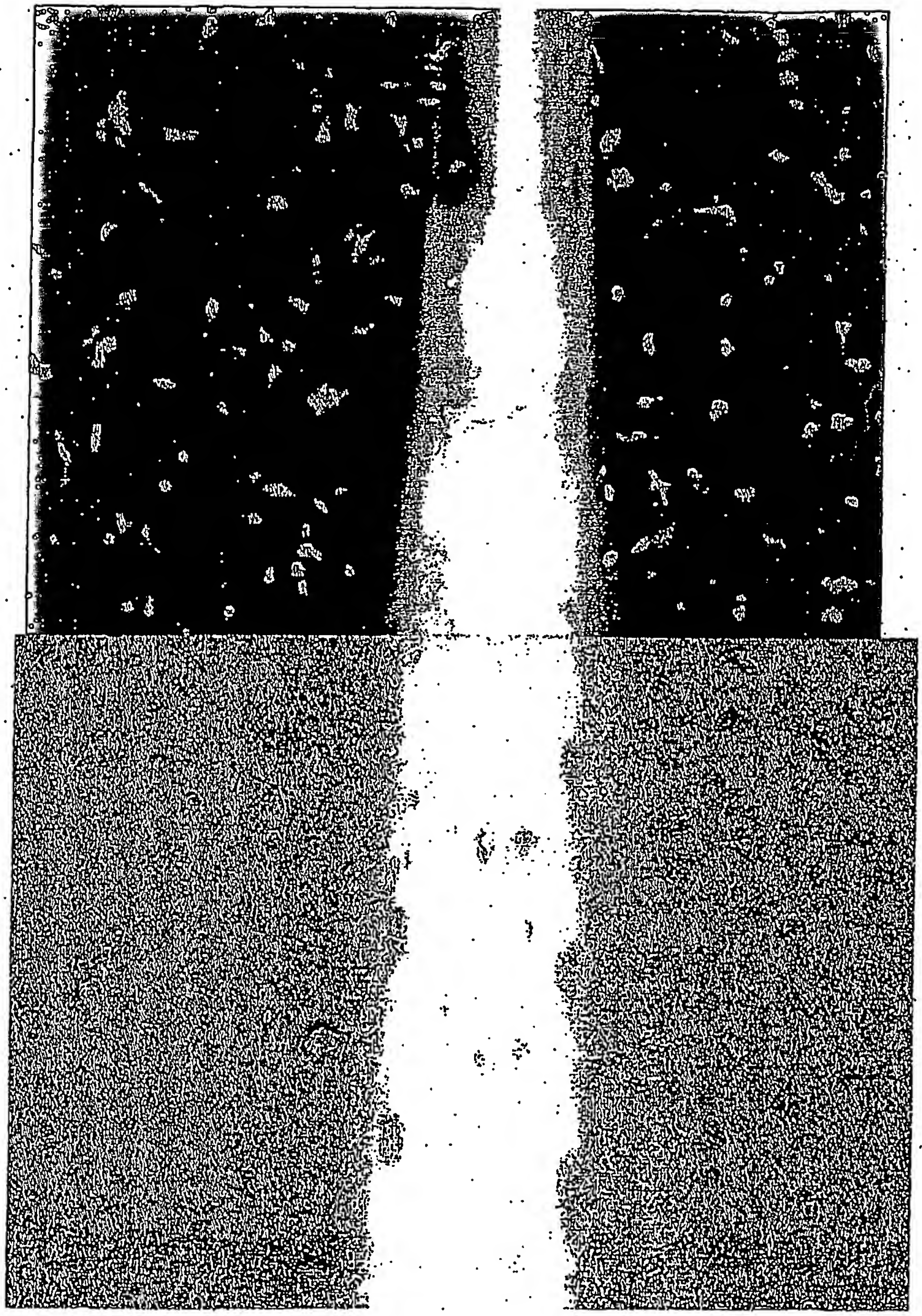

Transfection of C-26 cells with luciferase: comparison with FuGENE transfection reagent
450000
400000
350000
250000
200000
150000
100000
50000
0
RLU/
B5C/DOPE 2:1GFPra
B5C/DOPE 2:1GFPratio
Fugene 3:1 GFP

Fig. 9

Fig. 10



Transfection of NIH 3T3 cells ... **B3A/DOPE 2:1**

Table 1

Effect of the lipid (DOTAP)/protein (HN) ratio and cholesterol (Chol) on HN encapsulation efficiency

DOTAP/HN w/w ratio	DOTAP/Chol mole ratio	% HN encapsulation
300/1	1/1	93
100/1	1/1	90
50/1	1/1	90
30/1	1/1	88
10/1	1/1	79
3/1	1/1	35
100/1	1/0	90
100/1	1/1	92
100/1	2/1	89
100/1	4/1	80

Table 2
Serum, lung and nasal levels of HI, IgG1, IgG2a and IgA antibodies, and IFNγ levels produced by spleen cells, following i.n. vaccination with HN-loaded neutral, anionic and cationic liposomes

Group (n=5)	Vaccine[a]	Serum			Lung			Nasal			Spleen
		HI	IgG1	IgG2a	IgG1	IgG2a	IgA	IgG1	IgG2a	IgA	IFNγ (pg/ml)[b]
1		0	0	0	0	0	0	0	0	0	1800
2	F-HN	0	55	0	0	0	0	0	0	0	1400
3	Lip (DMPC)-HN (Neutral)	3±7 (0)	150	0	30	0	0	0	0	0	4200
4	Lip (DMPC/DMPG)-HN (Anionic)	6±13 (20)	500	0	40	0	0	0	0	0	4000
5	Lip (DC-Chol:DOPE)-HN (CAT1)	18±7 (0)	0	0	35	PBS	0	0	0	0	4900
6	Lip (DSTAP:Chol)-HN (CAT2)	28±29 (40)	20	0	20	0	0	80	0	0	2300
7	Lip (DDAB: Chol)-HN (CAT3)	136±32 (100)	100	0	80	30	0	30	0	0	3100
8	Lip (DOTAP: Chol)-HN (CAT4)	576±128 (100)	15000	730	1050	170	40	180	30	35	8000
9	Lip (DMTAP: Chol)-HN (CAT5)	672±212 (100)	30000	470	3000	30	15	80	70	25	7800
10	Lip (LLC: Chol)-HN (CAT6)	2368±1805 (100)	30000	9000	30000	1900	15000	30	300	50	10200
11	F-HN+CT (1μg)	1664±572 (100)	55000	7000	10000	1800	40	120	30	45	5200

Legend to Table 2

[a] The vaccine (3μg HN) was administered i.n. (10 μl per nare) on days 0 and 7. All liposomes (Lip) were prepared at a lipid/protein w/w ratio of 300/1; the molar ratio of cationic lipid/cholesterol (Chol) or DOPE (groups 5-10) was 1/1. Serum HI titer was tested on individual samples starting at 1/10 dilution; the data show the mean ± SD and % seroconversion (in parentheses). Antigen-specific isotypes were determined by ELISA on pooled samples starting at 1/20 dilution; the data show the means. Sera were tested 4 weeks and lung homogenates (pooled) and nasal washes (pooled) 6 weeks after the second vaccine dose.

[b] Spleen cells were incubated with the HN subunit preparation (10 μg/ml hemagglutinin) for 3 days. IFNγ levels in cell cultures without antigen were subtracted. In all groups, IL-4 levels were <20 pg/ml.

Table 3
The effect of the lipid/HN w/w ratio on the immunogenicity of HN-loaded cationic liposomes (CAT4, CAT5, CAT6)

No.	Vaccine[a] (n=5)	Lipid/HN w/w ratio	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFNγ (pg/ml)
1	F-HN		0	0	0	0	0	0	0	0	7430
2	Lip (DOTAP)-HN (CAT4)	300/1	496±295 (100)	15000	450	0	40	600	85	30	9780
3		100/1	196±119 (100)	5000	280	0	40	500	20	0	42220
4		30/1	36±50 (80)	1000	200	0	30	250	35	0	20440
5		10/1	28±18 (60)	600	30	0	20	250	0	0	20400
6		3/1	0	20	0	0	10	20	0	0	27780
7	Lip (DMTAP)-HN (CAT5)	300/1	388±260 (100)	2500	250	0	0	5500	200	1200	Not done
8		100/1	208±107 (100)	2200	600	0	0	7000	350	0	
9		50/1	130±118 (80)	850	150	0	0	4500	250	0	
10		30/1	48±71 (40)	450	0	0	0	1500	110	0	
11		10/1	24±35 (40)	120	0	0	0	500	0	0	
12	Lip (LLC)-HN (CAT6)	300/1	560±480 (100)	2000	1800	200	80	12500	3000	20000	
13		100/1	752±504 (100)	6500	6000	0	80	7000	5500	65000	
14		50/1	272±156 (100)	1900	700	0	40	5500	900	20000	
15		30/1	112±125 (80)	650	400	0	0	1500	200	0	
16		10/1	52±68 (40)	275	440	0	0	500	200	0	
17	F-HN+CT (1μg)	-	896±350 (100)	30000	8000	120	80	45000	2250	3000	
18	F-HN+Lip (DOTAP) (CAT4)	300/1	864±446 (100)	5000	1500	0	0	6000	500	1200	
19	F-HN+Lip (DMTAP) (CAT5)	300/1	320±226 (100)	1900	400	0	0	3750	225	1500	
20	F-HN+Lip (LLC) (CAT6)	300/1	704±525 (100)	30000	5000	500	80	35000	3000	80000	

[a] Data compiled from 2 separate experiments. In exp. 1 (groups 1-6), 3 μg HN, and in exp. 2 (groups 7-20), 2 μg HN was administered twice i.n. (10 μl per nare). In groups 18-20 the free antigen was mixed with performed liposomes in saline 30 min before administration. All liposomes were made with Chol at a cationic lipid/Chol 1:1 mole ratio. For details on assays, see Legend to Table 2.

Table 4
The effect of cholestrol (Chol) on the immunogenicity of HN-loaded CAT4, CAT5 and CAT6

No.	Vaccine[a] (n=5)	Cat lipid/ Chol w/w ratio	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFNγ (pg/ml)
1	F-HN	-	0	0	0	0	0	0	0	0	7430
2	Lip (DOTAP)-HN (CAT4)	1/0	320±0 (100)	15000	450	0	40	900	85	25	7480
3	Lip (DOTAP:Chol)-HN	1/1	496±295 (100)	15000	450	0	40	600	80	30	9780
4		2/1	168±216 (100)	7000	800	0	40	680	180	22	12870
5		4/1	195±111 (100)	15000	250	0	60	720	50	60	9330
6	Lip (DMTAP)-HN (CAT5)	1/0	320±188 (100)	20000	290	0	60	1000	40	0	8520
7	Lip (DMTAP:Chol)-HN	1/1	672±419 (100)	30000	300	0	120	3000	30	15	10900
8		2/1	576±368 (100)	25000	650	0	160	2500	160	200	8560
9		4/1	608±382 (100)	30000	600	0	80	4000	100	150	7490
10	Lip (LLC)-HN (CAT6)	1/0	2560±1568 (100)	30000	7000	100	640	30000	1500	9000	15550
11	Lip (LLC:Chol)-HN	1/1	2368±1805(100)	30000	9000	100	1280	30000	1900	15000	13780
12	F-HN+CT (1μg)	-	1664±572 (100)	55000	7000	20	20	10000	1800	1000	11110

[a] All liposomes were prepared at a lipid/HN w/w ratio of 300/1. The vaccine (3 μg HN/dose, 10 μl per nare) was administered i.n. on days 0, 7. For details on assays, see legend to Table 2.

Table 5
The effect of the instillation volume on the immune response induced by i.n. administraiton of HN-CAT5

Vaccine[a] (n=5)	Volume µl/nare/ dose	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFNγ (pg/ml)
PBS	10	0	0	0	0	0	0	0	0	20
F-HN	10	0	60	0	0	0	0	0	0	460
Lip (DMTAP:Chol, 1/1)-HN	5	180±123 (100)	7000	65	0	0	180	15	0	1240
	10	1248±1208 (100)	40000	1500	150	80	20000	150	700	18000
	25	1024±572 (100)	35000	800	35	80	1500	180	420	2700
	50	544±215 (100)	31000	325	0	80	1500	30	85	49000

[a] The vaccine was given on days 0, 7; each dose was divided between nares. For details on assays, see legend to Table 2.

Table 6
The effect of [redacted] on the immunogenicity of free and liposomal HN

No.	Vaccine (n=5)	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFNγ (pg/ml)
1	F-HN	0	50	0	0	0	0	0	0	460
2	F-HN+[redacted]	30±60 (20)	480	150	0	0	28	30	0	2000
3	F-HN+[redacted]	164±148 (80)	5000	1480	45	40	90	150	32	4940
4	F-HN+[redacted]	960±905 (100)	28500	6750	90	80	3100	700	280	13600
5	Lip (DI[redacted]	1248+1208 (100)	40000	2500	150	80	25000	150	700	18000
6	Lip (DI[redacted]	1088±893 (100)	30000	4500	150	60	1800	900	700	13000
7	Lip (DI[redacted] Lip (DI[redacted]	448±175 (100)	36000	600	40	60	1000	150	120	1440
8	Lip (DI[redacted]	1240±945 (100)	28000	8000	45	80	1000	7000	700	10400
9	F-HN+[redacted]	1340±730 (100)	35000	3000	190	20	3500	500	1000	12000

[a] HN (2 μg/do[redacted] administered i.n. (10 μl/nare/dose) on days 0, 7. All liposomes were prepared at a 300/1 (w/w) lipid/HN ratio; [redacted] (anionic liposomes); DMTAP/Chol, 1/1 mole ratio (CAT5). In group 8, HN+CpG-ODN were co-entrapped[redacted] n all groups, CpG-ODN (ODN 1018) dose was 10 μg. For details on assays, see legend to Table 2.

Table 7
Induction of cellular responses by cationic liposomes administered i.n.

No.	Vaccine[a]	Lipid/HN w/w ratio	% Cytotoxicity (Mean)[b] P815+peptide	P815	Proliferation[e] Δcpm (mean)
1	PBS		6[c]/5[d]	4[c]/4[d]	7009[f]/3493[g]
2	F-HN		8/4	5/4	7704/6959
3	CAT5-HN	300/1	16/8	13/7	10963/9119
4		100/1	9/4	9/4	12870/7353
5		50/1	3/2	3/2	17676/14831
6		30/1	3/0	2/0	17913/13742
7	CAT6-HN	300/1	4/0	2/1	20370/17624
8		100/1	21/13	7/6	24869/22014
9		50/1	6/5	3/3	20984/13010
10		30/1	8/7	5/4	11510/9699
11	F-HN+Empty CAT4	300/1	17/13	4/2	19392/15803
12	F-HN+Empty CAT5	300/1	17/13	7/6	11847/9519
13	F-HN+Empty CAT6	300/1	16/11	8/6	19269/15792
14	F-HN+CT (1μg)		10/5	9/3	28580/20858

[a] Mice were immunized on days 0 and 7. Splenocytes were harvested 6 weeks after the second vaccine dose. All liposomal formulations (groups 3-13) were made with Chol at a cationic lipid/Chol 1/1 mole ratio. In groups 3-10 the antigen was encapsulated in the liposomes; in groups 11-13 the free antigen was admixed with preformed empty liposomes.

[b] Measured in a 4h ^{51}Cr release assay at effector/target cell ratios of 100/1[c] and 20/1[d]. P815 were pulsed with the H2-HA2 189-199 peptide as described in the Materials and Methods.

[e] Splenocytes were incubated with 5 μg[f] or 0.5 μg[g] antigen per well for 72 h then pulsed with ^{3}H-thymidine for 16 h.

Table 8
Induction of humoral and cellular responses and protective immunity by HN-loaded cationic liposomes administered i.m. (x1), i.n. (x 1 or 2), or orally (x2)

No.	Vaccine[a] (n=5)	Route	Serum (n=10)				Lung (n=5)				Nasal (n=5)	Spleen (n=5)		Lung (n=5)
			HI	IgG1	IgG2a	IgA	HI	IgG1	IgG2a	IgA	IgA	Δcpm (mean)[b]	IFNγ (pg/ml)[c]	Virus titer (log 10)[d]
1	PBS		0	0	0	0	0	0	0	0	0	1641	0	7
2	F-HN	i.m. x 1	60±37 (70)	1000	40	0	0	80	0	0	0	1909	0	4
3		oral x 2	0	0	0	0	0	0	0	0	0	2253	0	ND
4		i.n. x 1	0	0	0	0	0	0	0	0	0	669	0	ND
5		i.n. x 2	0	55	0	0	0	70	20	0	0	2813	0	5
6	Lip (CAT4)-HN	i.m. x 1	424±141 (100)	21000	5500	0	40	900	500	0	0	3452	3300	0
7	(DOTAP/Chol)	oral x 2	0	0	0	0	0	0	0	0	0	0	1150	ND
8		i.n. x 1	40±28 (50)	450	80	0	0	50	20	0	0	482	1900	ND
9		i.n. x 2	409±172 (100)	25000	1300	60	120	10000	1000	350	45	8391	3200	0
10	Lip (CAT5)-HN	i.m. x 1	768±211 (100)	24000	8000	0	20	900	150	0	0	5632	0	1
11	(DMTAP/Chol)	oral x 2	0	0	0	0	0	0	0	0	0	553	0	ND
12		i.n. x 1	10±10 (0)	300	60	0	0	35	20	0	0	1277	0	ND
13		i.n. x 2	532±763(100)	10500	380	50	240	20000	700	2200	60	7331	3150	0
14	Lip (CAT6)-HN	i.m. x 1	864±1100 (100)	25000	10000	0	60	3500	900	0	0	6196	5750	0
15	(/Chol)	oral x 2	0	0			0	0	0	0	0	476	550	ND
16		i.n. x 1	34±50 (20)	1000	30	0	0	120	0	35	0	1705	6250	ND
17		i.n. x 2	2289±1576 (100)	25000	20000	400	360	30000	5000	20000	120	4912	15500	0
18	F-HN+CT (1ug)	i.n. x 2	756±650 (100)	21000	15000	20	240	22000	2500	1800	50	1933	5650	0

[a] Mice were immunized once or twice (days 0, 7) using 1, 2 and 4 µg HN/dose for i.m. (in 30 µl), i.n. (10 µl/nare) and oral (in 50 µl) administration, respectively. All liposomes were prepared at a lipid/HN w/w ratio of 300/1 with Chol at 1/1 mole ratio. Sera were tested separately (HI) or pooled (Elisa) at 4 weeks, and lung, nasal and spleen responses and protective immunity (all pooled) at 6 weeks post-vaccination.
[b] Δcpm = (mean cpm with antigen, 2.5 µg/well) – (mean cpm without antigen).
[c] IFNγ levels produced by spleen cells incubated alone were subtracted. 0 denotes <20 pg/ml.
[d] Tested 4 days after infection. See Materials and Methods for details. ND, not done.